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13. ABSTRACT (Maximum 200 Words)

We are investigating the effects of foods associated with reduced prostate cancer risk on a cooked meat carcinogen known to be associated with elevated cancer risk. Cooked muscle meats contain potent mutagens and carcinogens belonging to the heterocyclic amine class of compounds. One of these, PhIP, is a genotoxic carcinogen that has been shown to cause DNA damage in prostate tissue and prostate tumor formation in rats. We have developed a method to quantify urinary metabolites of PhIP in human volunteers that have been fed a meal of cooked chicken. Using this method, we have shown that PhIP metabolism may be affected by diet and lifestyle factors and that broccoli, soy, and tomatoes may influence the relative amounts of PhIP metabolite excretion. At the cellular level we investigated the metabolism of PhIP in human prostate cancer cells and investigated the relationship between DNA damage and gene expression. This research uses state-of-the-art analytical measurement methods to support conclusions about the role of diet and prostate cancer in humans. Although still preliminary, our results indicate that other components of the diet may have an effect on the metabolism of a commonly-occurring food carcinogen.

14. SUBJECT TERMS
Human metabolism, urinary metabolite determination, prostate cell culture, liquid chromatography/mass spectrometry, nutrition

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INTRODUCTION:

This study was designed to determine primary interventions that will prevent PhIP from causing prostate cancer. We investigated the effects of foods associated with reduced prostate cancer risk on a dietary carcinogen known to be associated with cooked meat and elevated cancer risk. Cooked muscle meats, a prominent component of the Western diet, contain potent mutagens and carcinogens belonging to the heterocyclic amine class of compounds. One of these, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a genotoxic carcinogen, causing mutations in bacteria [1] and mammalian cells in culture [2]. There have been several animal studies linking PhIP exposure to DNA damage in prostate tissue or prostate tumor formation [3-5]. In humans, prostate tissue has been shown to activate PhIP and DNA adducts have been detected in the tissue after metabolic activation [6].

PhIP is naturally formed in meats during the cooking process, with the highest levels found in grilled or fried meats. There are measurable amounts of PhIP in numerous foods, and in very well-done meats, PhIP can be found at levels up to 400 ng per gram of meat [7]. The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day [8]. We developed a method to quantify urinary metabolites of PhIP in human volunteers that have been fed a meal of cooked chicken. This method allows us to understand PhIP metabolism in humans and to measure the effects of potentially chemopreventive foods. At the cellular level we investigated the metabolism of PhIP in human prostate cancer cells as well as the effect of several of the putative active ingredients in the potentially chemopreventive foods.

Progress during the entire grant period:

TASK 1: Determine the stability of PhIP metabolism

A) Determine the stability of PhIP metabolism within an individual over time.

Three healthy, normal, male volunteers were recruited to participate in this phase of the study. The results of this study are described in a manuscript that was published Journal of Chromatography B [9]. A copy of this manuscript is included in the Appendix. This manuscript also describes the PhIP metabolite profiles of the 12 volunteers that have participated in the study to date.

B) Determine the assay variability of the same urine sample.

During the granting period, we performed repeated analysis of one urine sample to determine the stability of the metabolites over time (in urine frozen at -20°C) and the reproducibility of the LC/MS/MS method. We determined that the PhIP metabolites are relatively stable in frozen urine, with little to no degradation over time. The most labile with repeated freezing and thawing appears to be the N-hydroxy-PhIP-3-glucruonide.

We have implemented the precaution of freezing the deuterium-labeled spiking solution in small aliquots to prevent repeated thawing of the solution and this appears to adequately reduce the loss of this metabolite.

Sample variability continues to be an issue for the urine analysis. Variation exists in both repetitive injections of the same extraction and in repetitive extractions of the same sample. We believe that the primary factor contributing to the variation is the complex urine matrix itself, and we are continually updating our sample preparation procedures to attempt to reduce the interference from the urine matrix. Some of our attempts to minimize sample variation and improve the assay reproducibility are discussed in a manuscript published in Journal of Chromatography A [10].

Recent work to improve the method investigated recovery of the metabolites from the complex urine samples using new solid-phase extraction materials. The new mixed-mode solid-phase extraction cartridge "Focus" from Varian Sample Preparation Products was compared to "Strata" from Phenomonex. Recoveries were optimized for both, and we determined that the Focus product gave better recoveries for the N-hydroxy-PhIP-3-glucruonide and were about the same for other metabolites. Thus, the Focus column is now used routinely. We also evaluated the centrifugation step, trying to evaluate the clean-up gained for the overnight centrifugation using the filters with a 3000 molecular weight cut-off. We determined that the sample mass is about 1.5 milligrams total, but the amount retained by the filter was unable to be determined by our methods. Thus we have eliminated the step and still appear to have a robust method, but saving time and expense in sample preparation.

We have also changed the protocol to include the addition of deuterium-labeled internal standard for each of the metabolite peaks. This standard is the urine from a rat dosed with deuterium-labeled PhIP and contains the four metabolites we measure in the human urines. At this time, we extract each urine sample a minimum of three times and inject each extraction three times in order to obtain reliable data. Figure 1 shows a chromatogram from a typical urine sample with the addition of the pentadeutero-PhIP metabolite internal standards.

LC/MS was further optimized to better separate metabolites from the ion background and improve the column lifetime. The mobile phase gradient was made more isocratic and the solvent strength increased at the end of the run to remove bound material before the next run. These changes increased the run time, which includes re-equilibration, from 35 min to 47 min, but appears to make a more robust method. We continue to use columns we pack ourselves, which allows us to discard the packing material after 24 injections.

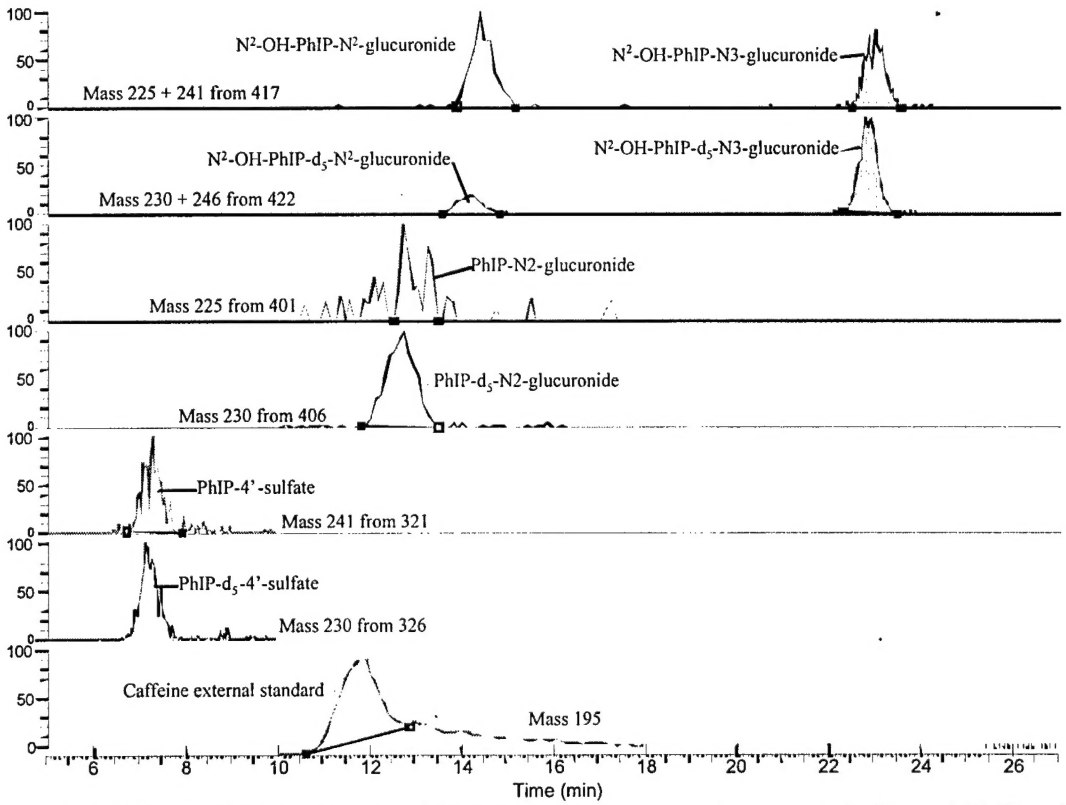

Figure 1. LC.VMS/MS chromatograms of the extract of human urinary metabolites of PhIP spiked with deuterium-labeled PhIP metabolites have parent and daughter masses 5 units greater than the natural metabolites.

TASK 2: Human Prostate Cells in culture

A) Effects of PhIP, N-OH-PhIP and 4'-OH-PhIP on cell proliferation

The effects of PhIP, N-OH-PhIP and 4'-OH-PhIP on cell proliferation in LnCAP and PC3 cells are shown in Figures 2 and 3. Cell proliferation is assayed with the CellTiter 96 Nonradioactive Cell Proliferation Kit (Pro-Mega) that measures cellular conversion of a tetrazolium salt into a blue formazan product. Cells are plated in 96-well plates and the absorbance of each well is determined spectrophotometrically at 595 nm. Absorbance read is directly proportional to cell number. PhIP and NOH-PhIP consistently stimulates cell growth 20-30% above controls in the androgen-sensitive prostate cancer cells, LNCaP (Figure 2), although NOH-PhIP becomes toxic at higher concentrations. There is no comparable effect in the androgen-insensitive PC3 cells. We are currently in the process of investigating potential mechanisms for this stimulation. We have found that PhIP binds to and activates the estrogen receptor in breast cancer cells, and that NOH-PhIP and PhIP isomers may have anti-estrogenic activity. We are planning to do parallel studies to determine if PhIP has a similar effect on androgen receptor activity.

In both prostate cancer cell lines, N-OH-PhIP is cytotoxic at concentrations above 0.3 µg/ml (Figures 2 and 3). This implies that at high concentrations, there is a build up of toxic intermediates; perhaps metabolites that are known to bind to DNA and cause cell death.

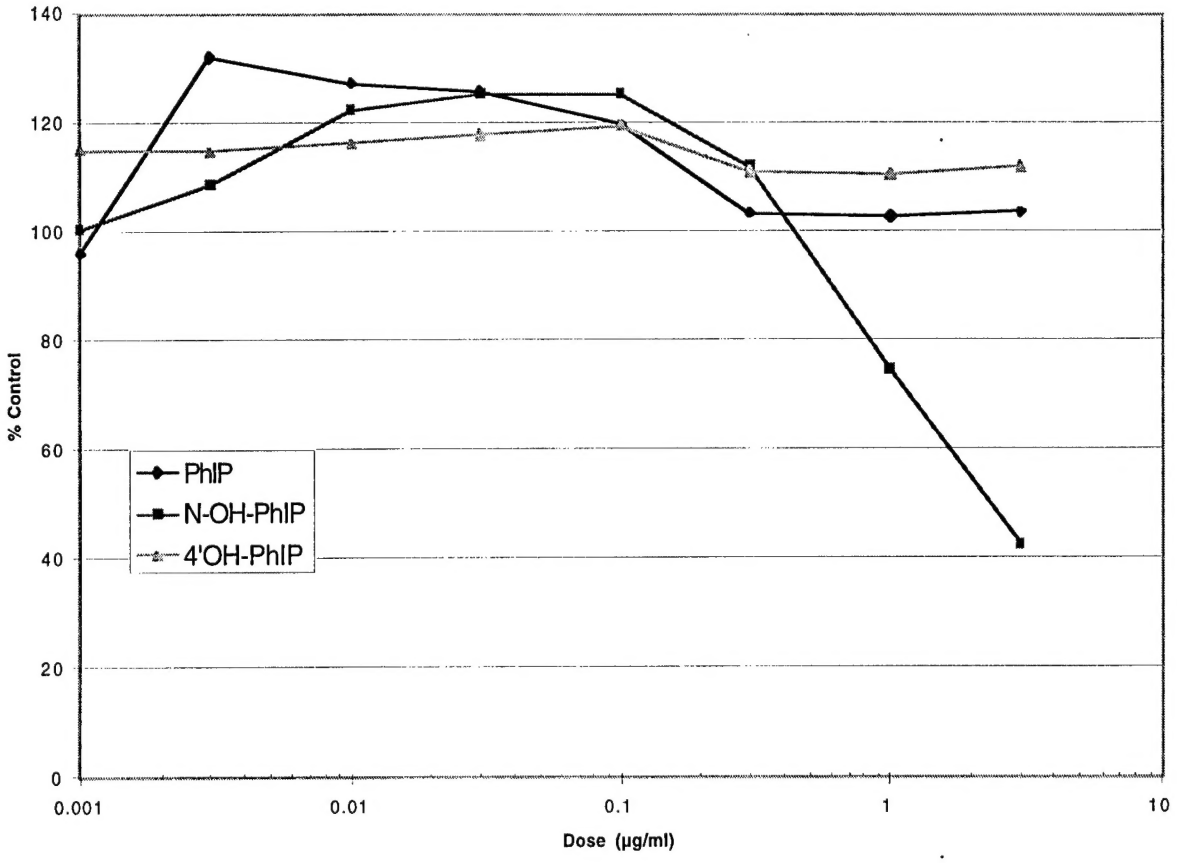

Figure 2. Effect of PhIP, N-OH-PhIP and 4'-OH-PhIP on cell proliferation of LNCaP cells.

In comparison, 4'-OH-PhIP does not appear to have an effect on prostate cancer cell proliferation, either stimulating cell growth or cell death. Based on our knowledge of the cytotoxic effects of the other heterocyclic amines in other cell systems and the effects of PhIP on the estrogen receptor in breast cancer cell lines, this implies that 4'-OH-PhIP is neither metabolized to a toxic intermediate nor activating the androgen receptor to stimulate cell growth.

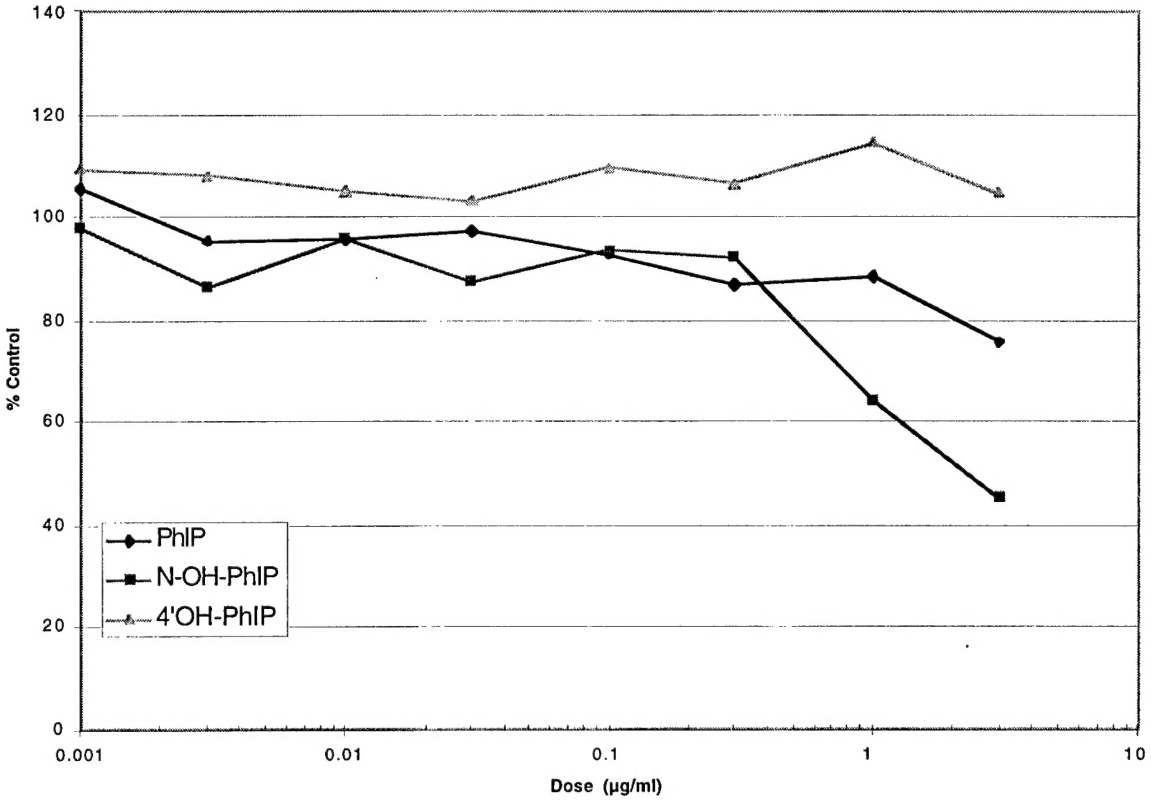

Figure 3. Effect of PhIP, N-OH-PhIP and 4'-OH-PhIP on cell proliferation of PC3 cells.

Task 2, B) Macromolecular binding

To more definitively determine the DNA binding of PhIP in prostate cancer cells, LNCaP cells were treated with C14-labeled PhIP and NOH PhIP and were harvested for accelerator mass spectrometry (AMS). AMS is a technique that measures attamole levels of [14C]-labeled agents with high precision. Our group at Livermore has successfully been able to study xenobiotic metabolism and DNA binding using this technology [11,12].

In this experiment cells were dosed with 0, 0.003, 0.03, 0.3 and 3.0 µg/ml PhIP and NOH PhIP and harvested 4 hours later by trypsinization. Cells were homogenized and homogenates were snap frozen. DNA was extracted for AMS analysis and mRNA was extracted and analyzed for gene expression using a commercially available human gene microarray. Cells were also analyzed in parallel for the effect of these levels of PhIP on cell growth.

Cell growth assays confirmed that PhIP stimulates cell growth in this androgen-responsive cell line (data not shown). Because these cells do not contain high

amounts of Phase I metabolizing enzyme activity, AMS analysis shows that PhIP treatment does not produce adducts that are significantly above background (Figure 4). In fact, four hours of 0.003 µg/ml PhIP treatment did not produce detectable adducts. However, LNCaP cells contain Phase II metabolizing enzymes that are able to further metabolize NOH-PhIP to an active intermediate. At the highest doses of NOH-PhIP, 4 hours treatment produced 1.7 x 10^7 adducts per 10^{12} nucleotides (Figure 4). Less adducts are found after 24 hours treatment because of the effects of DNA repair.

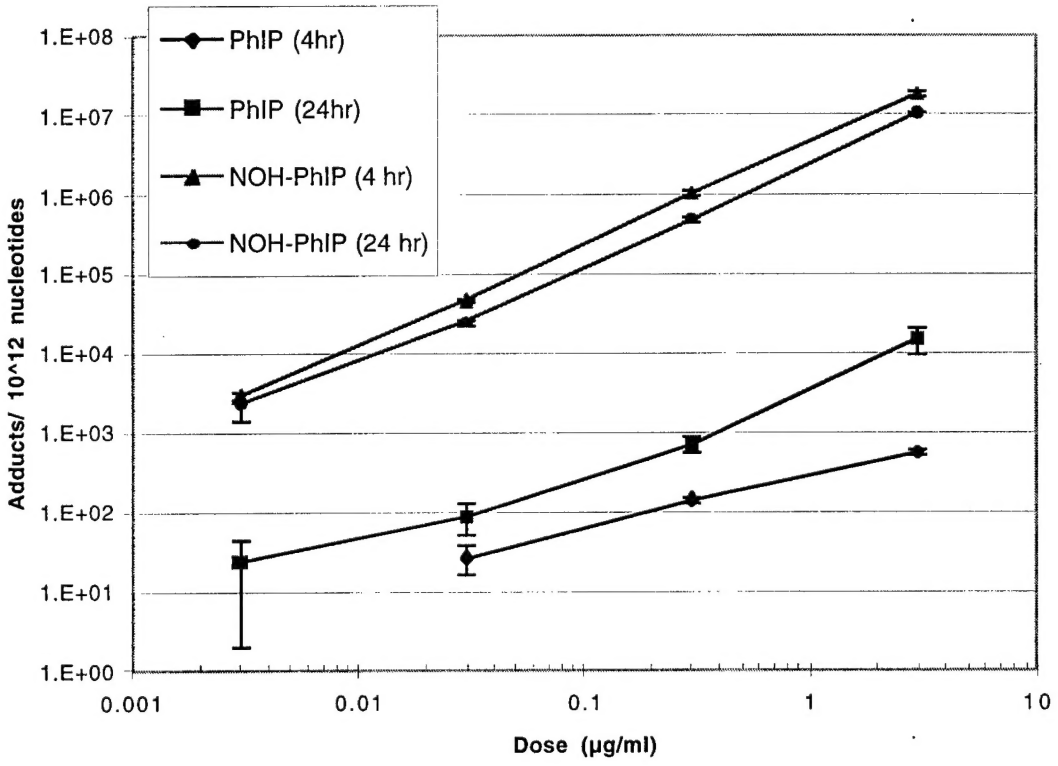

Figure 4. The effect of PhIP and NOH-PhIP on DNA adduct formation in LNCaP cells.

Gene expression analysis has been completed on the PhIP treated cells only. After 4 hours PhIP treatment there are 2 sets of genes that are changed: genes that are differentially turned on at different doses and genes that are turned on in a linear fashion with dose. In both of these sets of genes there are cell cycle control genes and cell signaling genes that will warrant further study. By 24 hours PhIP treatment, there is no evidence of any change in gene expression. This would suggest that PhIP stimulates early changes in gene expression that are not maintained throughout the exposure period.

After the NOH-PhIP gene expression data are fully analyzed, these data, along with the adduct levels and the cell proliferation data will be published in a peer-reviewed journal.

Task 2, C) Prostate cell metabolism

The metabolism of PhIP and NOH-PhIP was analyzed in years 1 and 2. Several candidate metabolites were found after NOH-PhIP treatment. The metabolism of 4'OH PhIP will not be assessed. Based on the cell proliferation data from this compound there is little evidence that these cells are competent to metabolize 4'OH-PhIP.

TASK 3: Link cellular metabolite profiles to urinary metabolite profiles

Linking cellular metabolites to urinary metabolite profiles proved problematic. Only one urinary metabolite was found to be produced by the cells. Excreted metabolites seem to be produced dominantly by the liver, and any extra-hepatic metabolites are found below the detection level in the urine.

TASK 4: Chemopreventive interventions

To investigate the effect of the intervention food on PhIP metabolism we quantify changes in PhIP urinary metabolites. In these studies, we fed the volunteers well-cooked chicken, collected urine and measured a baseline PhIP urinary metabolite profile. We then gave the subjects the intervention food daily for 3 days. On the fourth day we fed them chicken again and collected urine for another 24 hour period.

These results are described in 2 publications and a poster abstract, which are included in the Appendix.

A) Effect of tomatoes on PhIP metabolism in humans and in prostate cells

Four volunteers were recruited to participate in this study to date. The intervention food for this study was 1/2 c. commercially available pasta sauce daily at lunch for three days.

To provide the human volunteers with a higher dose of lycopene that is still representative of a typical diet, we examined the literature to find the best food source. Cooked tomato products have the most lycopene. We analyzed three tomato products using a spectrophotometric assay published by Rao et al. in 1998 and Arias et al in 2000 [13,14]. Three different samples of spaghetti sauce, Ragu Chunky Garden, Ragu traditional, and Prego Roasted Pepper were analyzed along with a negative control of a marinade sauce that contained no tomato products. These were extracted using hexane/acetone/methanol and the absorbance of the organic layer read in a spectrophotometer at 502 nm. All three tomato-containing sauces had lycopene, but not the marinade negative control. The Ragu Traditional sauce contained the most lycopene, about 30% more than the Ragu Chunky Garden and

about five times more than the Prego Roasted Pepper. Thus the Ragu Chunky sauce was fed to the volunteers.

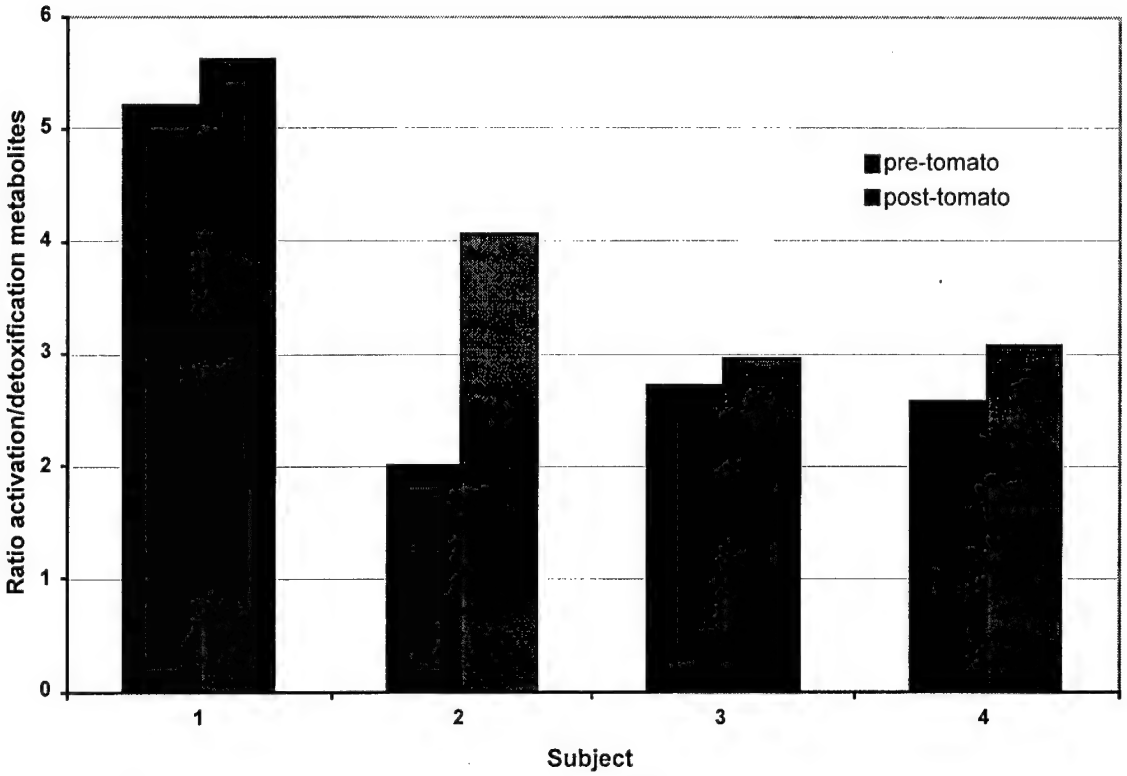

Figure 5. Effect of tomatoes on metabolite excretion. The ratio of the activation metabolites (N^2-OH-PhIP-N^2 glucuronide + N^2-OH-PhIP-N3-glucuronide) to the detoxification metabolites (PhIP-N^2-glucuronide+ 4'-PhIP-sulfate) is compared before and after the tomato intervention.

The analysis of the urine of the four men who participated in this study was detailed in Year 3 progress report. Our data showed a trend towards an increase in the activation metabolites (N^2-OH-PhIP-N^2 glucuronide and N^2-OH-PhIP-N3-glucuronide) compared to the detoxification metabolites (PhIP-N^2-glucuronide+ 4'-PhIP-sulfate) after the tomato intervention Figure 5. There was also a trend towards an increase in metabolite excretion in the 12-24 hour time period (Figure 6). Both of these results are similar to the effects that are seen with. However, the changes that we see are very small, the population is limited, so these results should be considered preliminary.

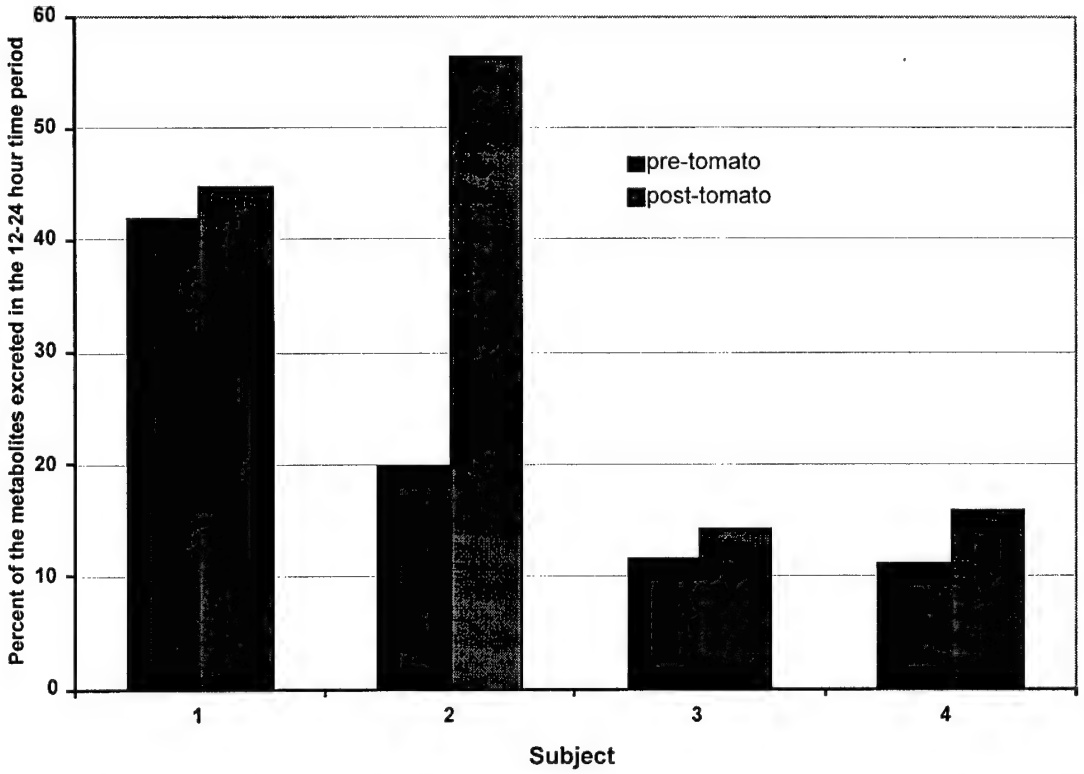

Figure 6. Effect of tomatoes on metabolite excretion rate. The percent of metabolites excreted in the 12-24 hour time period is compared before and after the tomato intervention.

B) Effect of soy on PhIP metabolism in humans and genistein in prostate cells

We have analyzed the urine from the seven volunteers who have participated in the soy intervention. In this trial the intervention food was a "soy shake" which contained 8 ounces of soy milk, 1 TBSP of a commercially available soy powder, bananas and honey. The shake was provided to the volunteers daily for 3 days. It appears that there is a trend toward an increase in the ratio of the activation metabolites (N^2-OH-PhIP-N^2 glucuronide and N^2-OH-PhIP-N3-glucuronide) compared to the detoxification metabolites (PhIP-N^2-glucuronide+ 4'-PhIP-sulfate). During Phase I metabolism PhIP is oxidized via cytochrome P4501A2 (CYP1A2) enzymes to a hydroxylated intermediate, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-hydroxy-PhIP). *N*-hydroxy-PhIP, which is itself mutagenic, can be converted to a biologically active form via Phase II metabolizing enzymes, primarily the acetyltransferases or sulfotransferases. This esterification generates electrophilic *O*-sulfonyl and *O*-acetyl esters, which have the capacity to bind DNA and cellular proteins [15-18]. PhIP can also be hydroxylated at the 4 position, forming 2-amino-1-methyl-6-(4'-hydroxy) phenylimidazo[4,5-*b*]pyridine (4'-hydroxy-PhIP), which is not mutagenic. 4'-hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted [19,20]. In addition, the parent compound can be directly glucuronidated at the N^2 and *N*3 positions. These glucuronides are not reactive and

this is believed to be a detoxification pathway [21,22]. Because *N*-hydroxy-PhIP is the first step of the activation pathway, we believe that the N^2-OH-PhIP-N^2-glucuronide and N^2-OH-PhIP-N3-glucuronide metabolites represent activation pathways metabolic products, whereas the PhIP-N^2-glucuronide and 4'-PhIP-sulfate represent detoxification pathways.

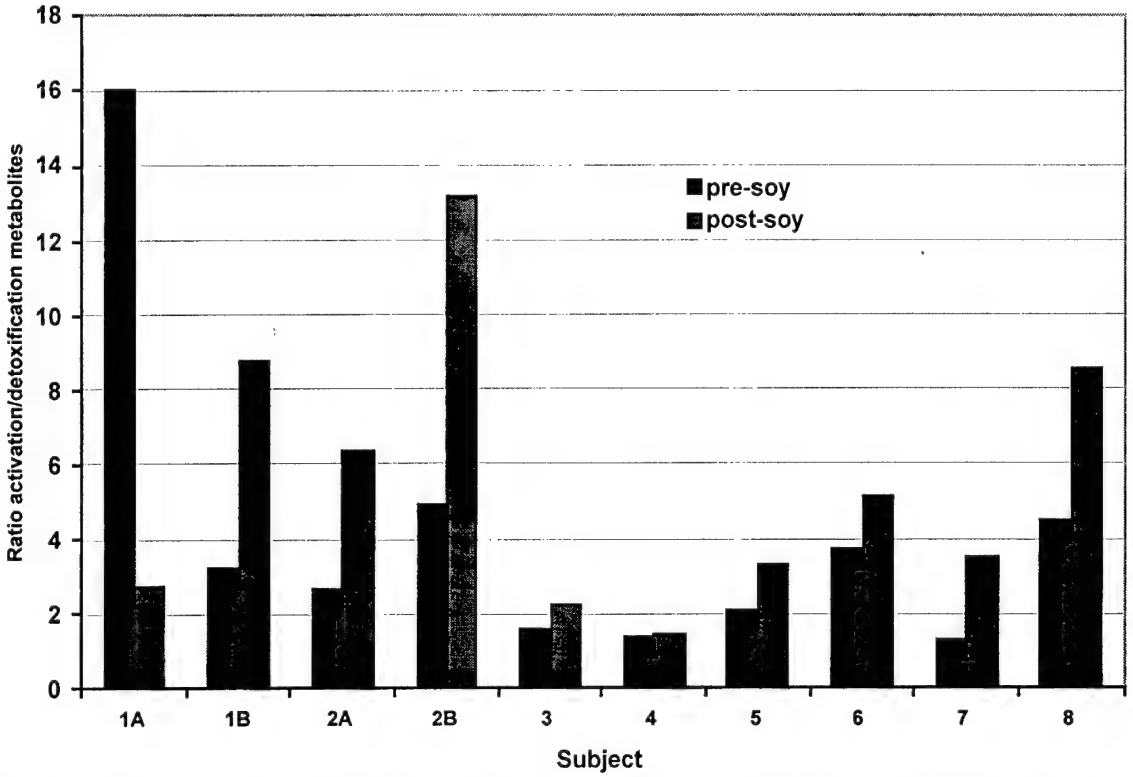

Figure 7. Effect of soy on metabolite excretion. The ratio of the activation metabolites (N^2-OH-PhIP-N^2 glucuronide + N^2-OH-PhIP-N3-glucuronide) to the detoxification metabolites (PhIP-N^2-glucuronide+ 4'-PhIP-sulfate) is compared before and after soy intervention.

With the exception of the first trial of Subject 1, the ratio of the activation metabolites to detoxification metabolites increased in all of the subjects.

Soy milk and soy powder are complex mixtures that contain a variety of biologically active substances; it is possible that one or several of the components in this mixture induce P4501A1, the enzyme responsible for N-hydroxylation of PhIP. Several recent studies have investigated the affect of soy protein on CYP protein expression and activity with varying results. Three studies showed an increase in protein expression or activity in CYP27B1, P4502A, CYP3A and CYP2B1 in various rodent models [23-25]. Another study in rats showed a decrease in mammary CYP1A1 activity and mRNA expression [26]. Another study of humans taking soy extract showed no inducibility of P4503A [27].

Soy also seems to affect the rate of metabolite excretion. With the exception of Subject 1, all of the subjects excreted more metabolites in the 12-24 collection period after the

soy intervention. The increase in metabolite excretion during the latter half of the collection period is driven primarily by an increased excretion of NOH metabolites (data not shown). It is possible that the induced activity of the P450 enyzmes demonstrated in Figure 4 also prolongs the metabolite excretion.

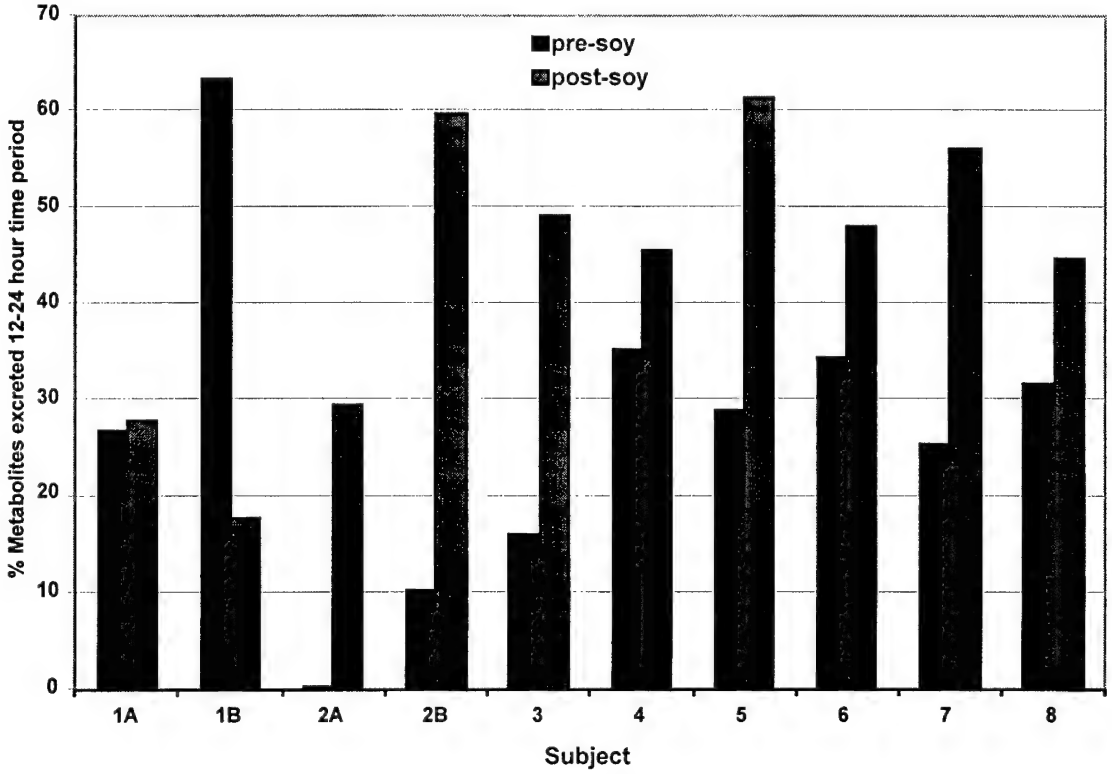

Figure 8. Excretion of PhIP metabolites in the 12-24 hour time period, before and after the soy intervention.

C) Effect of broccoli on PhIP metabolism in humans and sulforaphane in prostate cells

The progress on this task was discussed in the progress report for Year 1. We have published 2 papers that discuss these results, which are included in the Appendix.

KEY RESEARCH ACCOMPLISHMENTS:

Year 4:

- Determined that 4'-OH PhIP does not significantly effect cell proliferation in LNCaP and PC3 cells

- Determined that NOH-PhIP produces significant adducts in LNCaP cells and that relevant gene expression changes may occur after PhIP treatment

Year 3:

- Determined that PhIP metabolite excretion is not stable over time and may be highly dependent upon diet and lifestyle factors.
- Determined that rate of excretion is the most constant factor in individuals over time
- Determined that approximately 25% of the PhIP dose measurable in the chicken can be detected in the urine.
- Individuals that excrete metabolites more quickly tend excrete more metabolites
- Determined that soy and tomato consumption may change the ratio of activation/detoxification metabolites excreted.

Year 2:

- Determined that PhIP metabolism is not stable over time and may be highly dependent upon diet and lifestyle factors.
- Improved the sample preparation procedure to lower the impact of interfering substances in the urine and decrease the variation of LC/MS analysis.
- Determined that prostate cell metabolites differ from metabolites that we quantify in the urine.
- Determined that chrysin, a dietary flavonoid potentiates the cyctotoxicity of prostate cancer cells, but not other cell types.
- Determined that soy consumption may affect the relative amounts of PhIP metabolites excreted.

Year 1:

- Determined that PhIP and N-OH-PhIP can effect prostate and breast cancer cell growth.
- Solved problem of short HPLC column life by using laboratory packed columns that can be made inexpensively and the packing discarded frequently.

- Devised a sample preparation and analysis procedure to identify PhIP metabolites in prostate cancer cells, leading to understanding of metabolism of this prostate carcinogen in the target cells.

- Determined that broccoli may affect both the rate and relative amounts of PhIP metabolite excretion

REPORTABLE OUTCOMES:

Manuscripts:

J.S. Felton, M.G. Knize, L.M. Bennett, M.A. Malfatti, M.E. Colvin, K.S. Kulp, (2004) "Impact of Environmental Exposures on the Mutagenicity/Carcinogenicity of Heterocyclic Amines" *Toxicology*, 198:135-145.

K.S. Kulp, M.G. Knize, N. Fowler, C.P. Salmon, and J.S. Felton, "PhIP metabolites in human urine after the consumption of well-cooked chicken". *Journal of Chromatography B,* 802: 143-153.

M.G. Knize, K.S. Kulp, C.P. Salmon, G.A. Keating and J.S. Felton, (2002) "Factors affecting the human heterocyclic amine intake and the metabolism of PhIP". *Mutation Research*, 506:153-162.

J.S. Felton, M.G. Knize, C.P. Salmon, M.A. Malfatti, and K.S. Kulp. (2002)"Human Exposure to Heterocyclic amine Food Mutagens/ Carcinogens: Relevance to Breast Cancer". *Environmental and Molecular Mutagenesis,* 39:112-118

Knize, M.G., Kulp, K.S., Malfatti, M.A., Salmon, C.P., and Felton, J.S. (2001)"An LC/MS/MS urine analysis method to determine human variation in carcinogen metabolism". *Journal of Chromatography A*, 914:95-103.

Posters and Presentations:

Presentation: "Do heterocyclic amines influence cancer risk?" National Cancer Center, Tokyo Japan, Feb., 2004

Presentation: "Do heterocyclic amines influence cancer risk?" Osaka City University, Osaka Japan, Feb., 2004

J.S. Felton, K.H. Dingley, S.L. Fortson, P.T. Henderson, M.G. Knize, K.S. Kulp, N.P. Lang, M.A. Malfatti, D.O. Nelson, C.P. Salmon, and R.W. Wu "Heterocyclic amine

intake, bioaccessibility, and mechanisms of biological effects". American Association of Cancer Research Annual Meeting, March 27-31, 2004, Orlando Florida.

J.S. Felton, K.S. Kulp and M.G. Knize, "Mutagenic and carcinogenic chemicals in our diet". 4th International Conference of Environmental Mutagens in Human Populations, Florianopolis, SC, Brazil, May 4-8, 2003.

M.G. Knize, C.P. Salmon, K.S. Kulp, S.L. Fortson, and J.S. Felton, "Factors affecting heterocyclic amine intake". 2nd International Workshop on Analytical Chemical and Biological Relevance of Heterocyclic Aromatic Amines, Graz, Austria, May 7-9, 2003.

M.G. Knize, K.S. Kulp and J.S. Felton, "The effect of dietary soy protein on the metabolism of PhIP in humans" American Association of Cancer Research, Chemoprevention, Boston, MA, October, 2002

K.S. Kulp, M.G. Knize and J.S. Felton "Using human urinary PhIP metabolites to study individual variation in carcinogen metabolism and chemoprevention through dietary interactions" LLNL Science Day, September 2002

5/8/02 UC Davis Department of Engineering Seminar:
Technologies used in assessing risk from heterocyclic amines in cook food.

5/14/02 Sinai Medical and Cancer Center, NY, NY Seminar:
Human susceptibility to heterocyclic amines

9/24/02 Childrens Hospital Research Center, Oakland, CA Seminar:
Risk from heterocyclic amines in your diet

10/23/02 National Institutes of Health Staff Symposium on Diet and Health, Bethesda, MD, Overcooking of Meat and the Impact on your health

11/15/02 University of California Toxic Substance Research and Teaching Program, UC Riverside Symposium
Human susceptibility to heterocyclic amines

11/19/02 National Toxicology Program/National Institute of Health, Bethesda, Md
Presentation at hearing on risk of heterocyclic amines

1/18/03 AACR Symposium on Molecular Epidemiology, Kona, HA
Mutagenicity in humans of cooked foods.

4/29/03 California State University, Hayward, Course on Diet and Cancer
Diet and Cancer

5/15/03 Impact of the Environment on Colon Cancer Symposium, Miami Beach, Florida
Mutagens in Food

K.S. Kulp, M.G. Knize, S.L. McCutchen-Maloney, and J.S. Felton, "PhIP metabolites in human urine and human cancer cells: Implications for individual variation in carcinogen metabolism and chemoprevention" American Association of Cancer Research, San Francisco, CA, April 6-10, 2002

J.S. Felton, K.S. Kulp, M.G. Knize and S.L. McCutchen-Maloney, "PhIP metabolites in human urine and breast cancer cells: Implications for the study of individual variation of carcinogen metabolism and chemoprevention through dietary interactions" California Breast Cancer Research Program, March 8-10, 2002.

K.S. Kulp, M.G. Knize, S.L. McCutchen- Maloney, and J.S. Felton, "PhIP metabolites in human urine and human cancer cells: Implications for the study of individual variation of carcinogen metabolism and chemoprevention through dietary interactions" UC Davis Cancer Research Symposium; Sacramento, CA; October 2001

4/4/01 UC Berkeley Dept. of Epidemiology- Seminar (Risks of Overcooked Foods)

6/15/01 National Cancer Institute (Bethesda)- Seminar (Are Carcinogens in Food a risk for human Health?)

9/24/01 Environmental Mutagen Society Breast Cancer Conference- Symposium talk (Human Exposure to Heterocyclic Amine Food Mutagens/Carcinogens: Relevance to Breast Cancer)

11/12/01 8th International Conference on Carcinogenic/Mutagenic N-Substituted Aryl Compounds, Washington DC. (Factors affecting human heterocyclic amine intake and the metabolism of PhIP)

11/12/01 N-Substitute Aryl Compound International Meeting- Symposium talk (25 years of research on heterocyclic amines: What can we say about their impact on human cancer?)

3/2/02 Univ of Arkansas Colon Cancer Symposium- Symposium talk (Role of heterocyclic amines in colon and prostate cancer)

3/5/02 National Center for Toxicological Research- Seminar (Do Heterocyclic Amines pose a Human Risk)
Presentation "Development of Biomarkers for PhIP Metabolism", Department of Community, Occupational and Family Medicine, National University of Singapore, Feburary 28, 2001

Presentation "Heterocyclic Amines: Are they involved in human cancer?" Seminar for Biological Sciences Department, California State University, Stanislaus, March 9, 2001.

Presentation "Factors affecting heterocyclic amine intake and PhIP metabolism in humans", International Conference on Dietary Factors: Cancer Causes and Prevention, Vienna Austria, Feb 16, 2001.

Presentation "Development of Biomarkers for PhIP Metabolism", Department of Community, Occupational and Family Medicine, National University of Singapore, Singapore, Feburary 28, 2001

Presentation "Heterocyclic Amines: Are they involved in human cancer?" Seminar for Biological Sciences Department, California State University, Stanislaus, Turlock, CA, March 9, 2001.

Presentation "Factors affecting heterocyclic amine intake and PhIP metabolism in humans", International Conference on Dietary Factors: Cancer Causes and Prevention, Vienna, Austria, Feb 16, 2001.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" UC Berkeley Dept of Epidemiology, April 4, 2001.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" LLNL Biosecurity Facitliy, December 18, 2000.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" UC Davis, Cancer Center, October 6, 2000.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" CSU Deans of Science, November 3, 2000.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" University of Hawaii, Dept. of Epidemiology, January 18, 2001.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" University of Couth Carolina, Cancer Center, November 13, 2000.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" Children's Hospital, Oakland, CA, Mouse Research Unit, April 27, 2001.

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CONCLUSIONS:

This project has resulted in five published manuscripts, contributed to 38 posters or oral presentations and served as preliminary data for 2 funded studies.

This research used state-of-the-art instrument measurement methods to support conclusions about the role of diet and prostate cancer in humans. Although still preliminary, our results indicate that other components of the diet, such as broccoli, soy, and tomatoes may have an effect on the metabolism of a commonly-occurring food carcinogen. We have gained interesting insight into the stability of carcinogen metabolism in humans. Our investigations of the metabolism of PhIP and its intermediates and their effect on cellular response in prostate cancer cells may explain why this carcinogen specifically causes tumors of the prostate. It is possible that there are unique metabolic pathways present in prostate cells that produce a reactive intermediate that specifically causes DNA damage in the prostate.

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APPENDICES:

J.S. Felton, M.G. Knize, L.M. Bennett, M.A. Malfatti, M.E. Colvin, K.S. Kulp, (2004) "Impact of Environmental Exposures on the Mutagenicity/Carcinogenicity of Heterocyclic Amines" *Toxicology*, 198:135-145.

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Impact of environmental exposures on the mutagenicity/carcinogenicity of heterocyclic amines

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Abstract

Carcinogenic heterocyclic amines are produced from overcooked foods and are highly mutagenic in most short-term test systems. One of the most abundant of these amines, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), induces breast, colon and prostate tumors in rats. Human dietary epidemiology studies suggest a strong correlation between either meat consumption or well-done muscle meat consumption and cancers of the colon, breast, stomach, lung and esophagus. For over 20 years our laboratory has helped define the human exposure to these dietary carcinogens. In this report we describe how various environmental exposures may modulate the risk from exposure to heterocyclic amines, especially PhIP. To assess the impact of foods on PhIP metabolism in humans, we developed an LC/MS/MS method to analyze the four major PhIP urinary metabolites following the consumption of a single portion of grilled chicken. Adding broccoli to the volunteers' diet altered the kinetics of PhIP metabolism. At the cellular level we have found that PhIP itself stimulates a significant estrogenic response in MCF-7 cells, but even more interestingly, co-incubation of the cells with herbal teas appear to enhance the response. Numerous environmental chemicals found in food or the atmosphere can impact the exposure, metabolism, and cell proliferation response of heterocyclic amines.

Keywords: Mutagenicity; Carcinogenicity; Heterocyclic amines; PhIP

1. Introduction

Well-done cooking of muscle meats results in the natural formation of heterocyclic aromatic amines that have been found to be potent mutagens in various assay systems. These same compounds produce tumors at multiple organ sites in both male and female mice and rats (Shirai et al., 1997; Sugimura, 1997). Furthermore, 100% of non-human primates given one of these heterocyclic amines (2-amino-3-methylimidazo[4,5-*f*]quinoline; IQ) developed hepatocarcinomas after a very short latency (Adamson et al., 1990, 1994). More than two-thirds of human epidemiological studies (both case-control and cohort) correlating meat intake and cooking practices have shown an increased risk of cancer for individuals that

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prefer well-done meat. These epidemiology studies identify breast, colon, stomach, lung and esophagus as the primary target organs. For human prostate cancer, more studies are needed to determine if meat intake is correlated with the disease. Several authors have described cooking methods such as lower temperature frying (Knize et al., 1994; Skog et al., 1995), pre-microwaving (Felton et al., 1994), marinating (Salmon et al., 1997), and frequently turning the meat during frying (Salmon et al., 2000) that markedly reduce the HA levels in foods.

Schwab et al. (2000) wrote a comprehensive review of compounds that may inhibit the genotoxic/carcinogenic effects of HAs. They compiled data from more than 150 reports that described more than 600 agents that attenuated the effects of HAs. Most of the agents were evaluated in *Salmonella* TA98 mutation assays, but aberrant crypt foci, liver foci and DNA adducts were also used to assess in vivo effects. Protection was presumed to be a result of a number of different mechanisms, including (1) HA inactivation by a number of different mechanisms by compounds like chlorophyllin, (2) inhibition of the activating cytochrome P4501A family of enzymes, (3) induction of detoxifying enzymes, or (4) enhancement of DNA repair mechanisms. This report will not attempt to re-review these studies, but give an account of new literature studies and those primarily ongoing in our laboratory, including those which show an enhancement (increased risk) rather than an inhibition of HA effects.

We have known for many years that HAs are metabolized by cytochrome P450 1A1 and 1A2 to an N–OH intermediate (Holme et al., 1989; Kaderlik and Kadlubar, 1995) and that further metabolism by a series of conjugating enzymes is either further activating or detoxifying, depending on the specific compound (see Fig. 1). It is reasonable to suspect that any polycyclic aromatic hydrocarbon, flavonoid, or other environmental inducer that changes the activity of the cytochrome P450 enzymes would impact the kinetics of HA metabolism. What is less well understood is

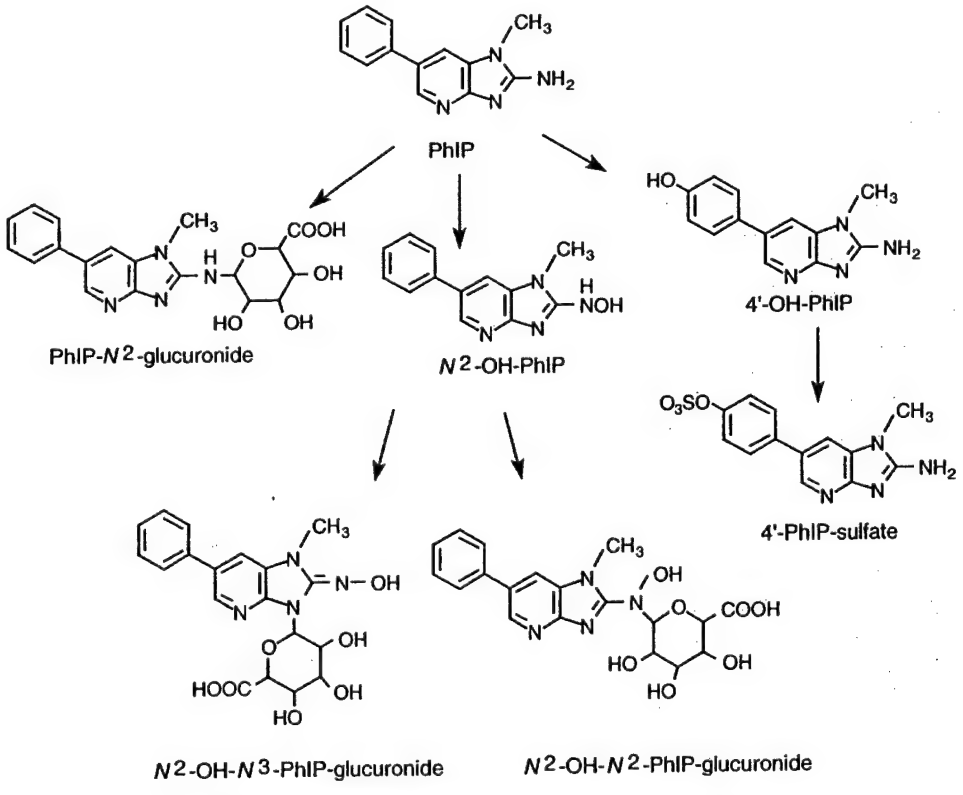

Fig. 1. Pathways for the activation and detoxification of PhIP.

the effect of competition at the CYP450 or other enzymatic active-site by these, and other, environmental compounds.

Gooderham et al. (2002) described an increased estrogenic response in cells exposed to PhIP. We have found a similar response in MCF-7 human breast cancer cells. This important finding may help explain why PhIP behaves as a complete carcinogen in rat breast tumorigenesis. Clearly, estrogenic behavior should increase cell proliferation and make the mammary cells quite susceptible to PhIP genotoxicity.

Given these very interesting data, it is important to determine the extent to which these HAs show enhanced mutagenicity or carcinogenicity when environmental compounds are given prior to or at the same time as the heterocyclic amines are consumed.

1.1. Heterocyclic amine exposure

Extensive analysis of many different kinds of foods has demonstrated the presence of heterocyclic amines in muscle meats cooked to a well-done state. Table 1 shows selected data for types of commonly consumed beef in North America. These data were obtained from meats cooked "well-done" in local restaurants. In the samples, MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline), PhIP, and IFP (2-amino-(1,6-dimethylfuro[3,2-*e*]imidazol[4,5-*b*]pyridine) are found at levels that vary from below the level of detection (about 0.1 ng/g) to 19 ng/g. Studies of the amounts of HAs produced in foods as a result of regional cooking practices are reported for Great Britain (Murray et al., 1993), Sweden (Johansson and Jägerstad, 1994; Skog et al., 1997), Switzerland (Zimmerli et al., 2001), Spain (Busquets et al., 2004) and Japan (Wakabayashi et al., 1993). In most cases PhIP and MeIQx tend to be the most abundant HAs, but the levels are very dependent on the cut or muscle origin of the meat product and the cooking method.

Table 1
Heterocyclic amines are found in various quantities in restaurant-cooked meat (ng heterocyclic amine per g cooked meat)

Sample	Restaurant (doneness)	IFP	MeIQx	PhIP
Top loin steak	A (well done)	7.0	1.3	7.7
Top loin steak	B (well done)	nd	1.3	0.86
Flank steak	C (well done)	8.2	1.9	19
Prime rib	C (well done)	nd	nd	nd
Beef (fajitas)	D (unspecified)	1.4	0.93	1.7

nd: not detected.

1.2. Metabolism in rodents and humans

In mouse liver microsomes, PhIP is metabolized to two major metabolites, one of which is the direct-acting mutagen, N–OH-PhIP. The other major mouse metabolite has a hydroxylation at the 4′ position of the phenyl ring and appears to be a detoxification product (Turteltaub et al., 1989). In rat hepatocytes PhIP is transformed to 4′-OH-PhIP as the primary product, followed by glucuronidation and sulfation of this metabolite (Langouet et al., 2002; Malfatti et al., 1994). Glucuronide and sulfate conjugates of 4′-OH-PhIP have been detected in human hepatocytes, but as minor metabolic products (Langouet et al., 2002). Thus, it is important to understand factors that favor formation of one or the other of these metabolites, as the ratio will affect the level of reactive intermediates available for DNA binding (adduct formation) and mutation. Other activation pathways besides P450 hydroxylation appear to be present in the mouse. Studies of transgenic CYP1A2 null mice demonstrated that PhIP was equally potent in causing lymphomas and tumors of the lung and liver in the mice lacking CYP1A2 as in wild type mice (Kimura et al., 2003). DNA adduct levels were reduced in the knockout mice, suggesting that the level of adducts is not directly correlated with the formation of blood, liver or lung tumors in this system (Snyderwine et al., 2002).

In humans, cytochrome P4501A2 activation of the parent amine to the corresponding 2-hydroxyamino intermediate is the predominate step. As Fig. 1 shows, conjugating reactions can occur, impacting the overall metabolism of the HA (Buonarati et al., 1990; Turteltaub et al., 1989). For PhIP, the *N*-hydroxy intermediate can be esterified by sulfotransferase and/or acetyltransferase to generate the highly electrophilic *O*-sulfonyl and *O*-acetyl esters (Buonarati et al., 1990). But unlike the rodent, human PhIP metabolism is dominated by glucuronidation. Both the N^2 and the N^3 positions of PhIP are glucuronidated directly (most likely these are non-reactive intermediates) and glucuronidation of *N*-hydroxy PhIP intermediates can be envisioned as a direct detoxification pathway (Malfatti and Felton, 2001).

Table 2
Recent reports of foods or contaminants shown to affect HA outcomes in vitro or in vivo

Food/exposure	Effect	Reference
Green, black, white tea	Inhibit various enzymes, Prevent DNA binding	Santana-Rios et al. (2001), Krul et al. (2001), Muto et al. (2001), Lin et al. (2003), Lin et al. (2003)
Cruciferous vegetables	Induce GST, P4501A2	Steinkellner et al. (2001), Murray et al. (2001)
Wheat bran fiber	Lignin binding	Yu et al. (2001)
Coffee	Induce glucuronosyl and glutathione transferases	Huber et al. (2002)
Milk products	Binding to bacteria	Knasmuller et al. (2001)
Cigarette smoke	Induce CYP1A2	Mori et al. (2003)
Fat	Increase intestinal tumors in rat	Ubagai et al. (2002)
Organophosphate insecticides	Enhances mutagenic response	Wagner et al. (2003)

1.3. Foods affecting HA damage

Table 2 reviews studies demonstrating that many foods have the potential to affect the mutagenic/carcinogenic effects of the heterocyclic amines, by a variety of mechanisms. These data represent studies published since the review by Schwab et al. (2000). With complex mixtures the exact mechanism and chemical or chemicals responsible for the effect are frequently unknown. But in some cases, specific food components are known that decrease the levels of DNA adducts, suggesting a possible protective effect for cancer initiation. DNA adducts can be relevant biomarkers of cancer risk, but as stated above, at least in the mouse, they may not always be predictive for these tumor sites. Table 3 shows that a variety of chemicals in food reduce PhIP-DNA adducts in rodents and in many cases at the site of tumorgenesis for that species. In contrast, caffeine increased colon adducts in the rat after PhIP exposure (Takeshita et al., 2003). Much more needs to be done to understand the impact of dietary interactions, competing substrates and adduct levels on tumorgenesis.

1.4. Human studies with broccoli

Identification of the four major metabolites of PhIP in humans allowed our laboratory to develop LC/MS/MS detection and quantification methods for these metabolites in urine after a single meal of cooked-well-done chicken (Kulp et al., 2000, 2003). Measuring changes in the amounts of total metabolites excreted or the rate of excretion of specific metabolites with time can be used to understand individual differences in metabolism, as well as whether environmental agents can impact the metabolism of PhIP.

Using this assay, we are now doing intervention studies to determine whether environmental chemicals

Table 3
Food components reducing PhIP-DNA adducts in rodents

Food component	Target tissue	Reference
Chlorophyllin	Colon	Guo et al. (1995)
Indole-3-carbinol	Colon	Guo et al. (1995)
	Mammary, colon, liver	He et al. (1997)
	11 of 12 tissues reduced	He et al. (2000)
Conjugated linoleic acid	Liver and mammary gland	Schut et al. (1997), Futakuchi et al. (2002)
Reservatrol	Mammary gland	Dubuisson et al. (2002)
Omega-3 fatty acids	Liver spleen small intestine in mouse, not rat	Josyula et al. (1998)
Quercitin, genestein, tangeretin or β-naphthoflavone	Colon	Breinholt et al. (1999)
Docosahexaenoic acid	Colon	Takahashi et al. (1997)

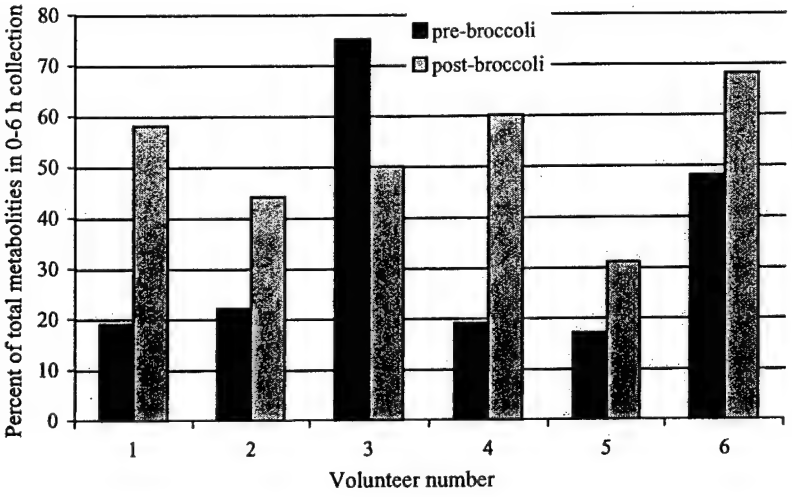

Fig. 2. Effect of broccoli on the percent of PhIP metabolites excreted in the urine 0–6 h after consuming well-cooked chicken. Black bar represents percent of total PhIP metabolites excreted during the 0–6 h collection time before the broccoli intervention. Grey bars represent excretion during the same time period after the intervention.

such as isothiocyanates and/or indol-3-carbinols in broccoli and other cruciferous vegetables can impact PhIP metabolism. In a preliminary study, six healthy men were fed a single meal of well-done chicken after abstaining from broccoli or related cruciferous vegetables for 3 days. Metabolites were determined in urine collected in 6 h increments. After eating cooked broccoli for 3 days, the protocol with well-done chicken was repeated. We found that the percent of the total metabolites excreted in the first 6 h after chicken consumption was increased in all but one individual after the broccoli intervention (Fig. 2). This suggests chemicals in the broccoli increase the metabolic rate for PhIP metabolism, possibly by increasing the oxidation by P4501A2.

Murray et al. (2001) fed a group of 20 volunteers broccoli and brussels sprouts and showed induction of CYP1A2 activity and a decrease of the parent MeIQx and PhIP in the urine. They did not measure the metabolites, but the finding is consistent with that from our laboratory.

1.5. Computational simulation of environmental chemical effects

To understand the role of compounds that either induce enzyme activity or compete at the P450 active site we have begun to model the active site of human cytochrome P4501A2. This is possible by homology modeling the measured structure (X-ray diffraction derived coordinates) of other P450s from rabbit and bacteria. By calculating the molecular dynamics of the docking of the small molecules into the active site we can correlate mutagenic activity of various HAs and at the same time get comparative active-site-binding parameters (Colvin et al., 1998; Sasaki et al., 2002).

1.6. Inhibition of mutagenic activity in bacteria by flavonoids

We were able to compare the inhibition of PhIP and MeIQx mutagenicity by co-incubating the HAs with two flavonoids, naringenin and apigenin, in an Ames *Salmonella typhimurium* TA98 assay (Fig. 3). Interestingly, although they are structurally very similar, they produced a different inhibitory response. Apigenin was able to inhibit the mutagenicity of PhIP by 90% and MeIQx by 69%. Narigenin, which differs by a single double bond, was only able to inhibit PhIP mutagenicity by 16%, although the compound was more potent in the presence of MeIQx, inhibiting mutagenity by 44%.

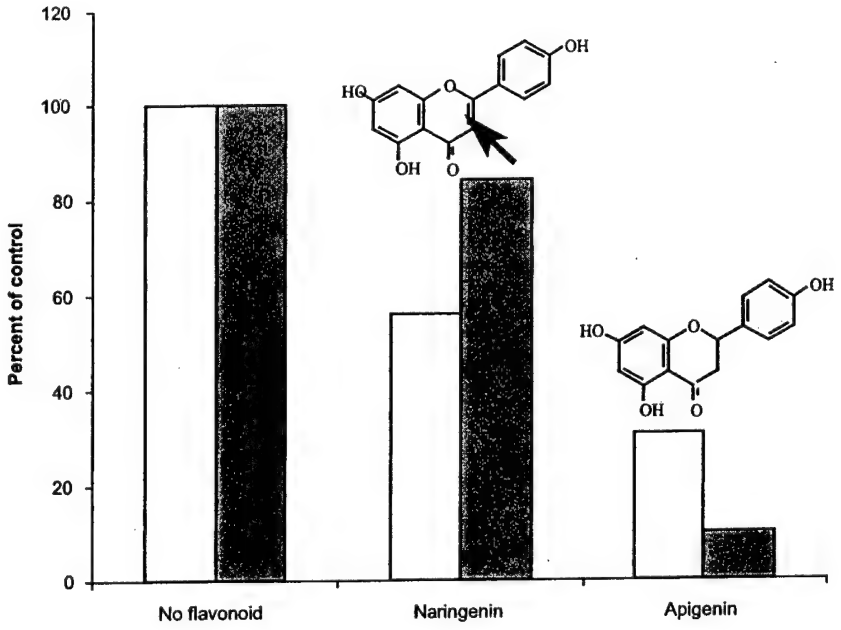

Fig. 3. Inhibition of mutagenic activitiy of MeIQx (open bar) and PhIP (filled bar) activity in *Salmonella typhimurium* TA98. Apigenin and naringenin differ in only a single double bond, indicated by an arrow, yet apigenin is a more potent inhibitor for both MeIQx and PhIP. Both flavonoids were used at 15 nmol/plate.

1.7. Impact of a dietary supplement on PhIP induced mutagenicity, cell proliferation and estrogen response

Fifty percent of North Americans use some form of dietary supplement, natural product, or alternative therapy (Ernst and Cassileth, 1999; Kaegi, 1998; Smith and Boon, 1999). People diagnosed with cancer are motivated to try alternative products because they do not want to leave unexplored any option that could treat their cancer, prevent recurrence, or improve their quality of life (Richardson et al., 2000). The literature on dietary supplements is widely disseminated in the lay press (Percival, 1994; Stainsby, 1992; Whitaker, 1995) and on web sites where information, presented as factual, is rarely backed by scientific investigation (Stainsby, 1992). The consumers choosing to use these products are not only emotionally vulnerable to spending money on expensive products that may or may not have value, but as cancer survivors with increased risk of recurrence, may be physically vulnerable to further exposure. We are interested in understanding how breast cancer survivors who take these products while continuing to eat well-done meat may be impacting their health.

Flor-Essence® Herbal Tea is a widely consumed herbal tonic, available at health food stores and on the internet in North America and other sites world-wide. The product is a mixture of eight herbs, including burdock root (*Arctium lappa*), sheep sorrel (*Rumex acetosella*), slippery elm (*Ulus rubra*), Turkish rhubarb (*Rheum palmatum*), watercress (*Nasturt officinale*), blessed thistle (*Carduus benedictus*), red clover (*Trifolum pratense*) and kelp (*Laminaria digitata*). Individually, these herbs have been shown to have anti-carcinogenic, estrogenic, anti-estrogenic, and anti-oxidant properties, among others.

We evaluated Flor-Essence® in the Ames test to determine if the anti-mutagenic activity described by others for one of its components, Turkish rhubarb (Horikawa et al., 1994; Lee and Tsai, 1991), was detectable in this complex mixture. Using the *S. typhimurium* strain TA98, we determined that Flor-Essence® tea inhibits the mutagenic activity of both PhIP ($P = 0.04$) and IQ ($P = 0.002$) in a dose-dependent manner (Fig. 4). In contrast,

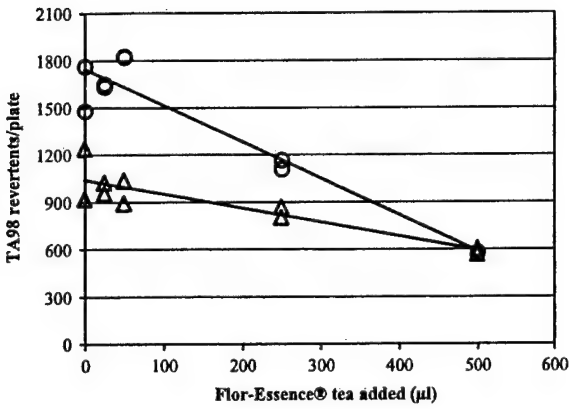

Fig. 4. Effect of Flor-Essence® on PhIP (triangle) and IQ (circle) induced mutagenicity using *Salmonella typhimurium* strain TA98 to test Flor-Essence® for anti-mutagenic activity. Flor-Essence® herbal tonic inhibits the mutagenic activity of PhIP and IQ in a dose dependent manner. The slope of each line is significant IQ ($P = 0.002$) and PhIP ($P = 0.04$).

Flor-Essence® did not inhibit the mutagenicity of DMBA or benzo[*a*]pyrene using the TA100 *S. typhimurium* strain (data not shown). TA 100 is a bacterial strain that is sensitive to base substitutions, and is commonly used to quantify DMBA and benzo[*a*]pyrene mutagenicity. These results suggest that Flor-Essence® may be inhibiting pathways that are important for PhIP activation, while having no effect on those important for DMBA activation.

N–OH-PhIP causes cytotoxicity in LNCaP and PC3 prostate cells at concentrations above 1 µg/ml. Adding Flor-Essence® herbal tonic with the N–OH-PhIP protects prostate cancer cells from cytotoxicity (Fig. 5). Cell toxicity was measured using the Cell Titer 96 Nonradioactive Cell Proliferation Kit. The results from this experiment suggest that Flor-Essence® herbal tonic may prevent the formation of DNA adducts known to be related to N–OH-PhIP-induced cytotoxicity. Taken together, these experiments suggest that Flor-Essence® can influence PhIP-induced DNA damage in both bacterial and mammalian cells.

We have been investigating the estrogenic response in MCF-7 human breast cancer cells after PhIP exposure using computational, analytical and biochemical tools. Early data shows that PhIP induces a weak, but significant, dose-dependent activation of this response in these cells. Interestingly, Flor-Essence® herbal tonic, which is highly estrogenic in this assay system, is able to enhance the estrogenic response caused by PhIP in these cells. Fig. 6 shows the effect of Flor-Essence® and PhIP on estrogen receptor activation in MCF-7 cells. Estrogen receptor activation is measured using a standard estrogen responsive reporter plasmid containing three vitellogenin estrogen responsive elements (EREs) upstream of the luciferase

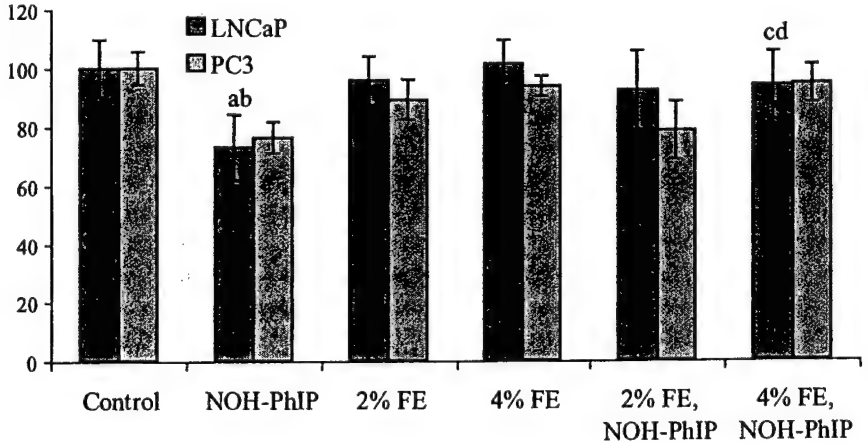

Fig. 5. PhIP-induced toxicity in LNCaP and PC3 prostate cell lines is inhibited by 2 and 4% Flor-Essence® administration. Cells were treated with 1 µg/ml NOH-PhIP, 2% or 4% Flor-Essence®, or 1 µg/ml NOH-PhIP with 2% or 4% Flor-Essence® added. In both LNCaP and PC3 cells 4% Flor-Essence® protects from the cytotoxic effects of NOH-PhIP: (a) significantly less than control, $P < 0.05$; (b) significantly less than control, $P < 0.01$; (c) significantly greater than NOH-PhIP alone, $P < 0.05$; (d) significantly greater than NOH-PhIP alone, $P < 0.01$.

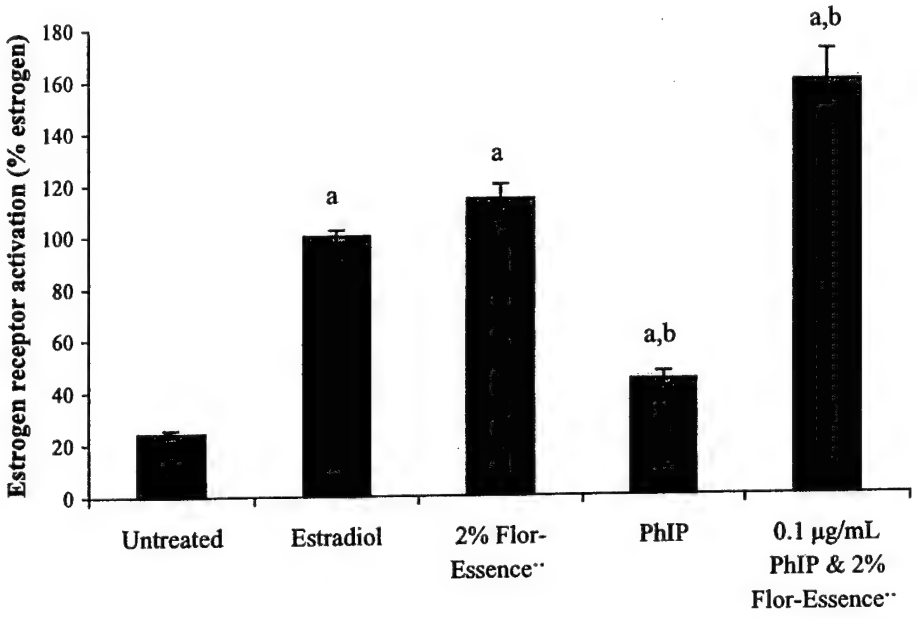

Fig. 6. Effect of PhIP and Flor-Essence® on estrogen receptor activation in MCF-7 human breast cancer cells. Data are presented as percentage of the response of estrogen. Error bars represent standard error of the mean: (a) significantly greater than control, $P < 0.01$; (b) significantly different than estradiol, $P < 0.01$.

reporter gene. Flor-Essence tea at 2% concentration gave a 110% response that was not significantly different than physiological concentrations of estrogen. When the tea and PhIP were co-administered the estrogen activation was 150%. This was additive for the two compounds and clearly higher than estrogen alone. More work will need to be done to understand the ramifications of strong genotoxic agents like PhIP that may also have an effect on cell growth. Our experiments investigating the effects of an herbal supplement on PhIP exposure are an excellent example of how being exposed to complex mixtures of chemicals may have a competing effect on the ultimate risk of cancer development; in this case we see both protection and enhancement of tumorgenesis. The ultimate understanding of the effects of exposure to complex mixtures will come after we learn much more about the details and kinetics of the competing pathways.

2. Conclusions

The impact of environmental exposures on the mutagenicity and carcinogenicity of heterocyclic amines has been shown to be significant and real. Up regulation of the cytochrome P4501A2 levels is not unexpected, as we use enzyme inducers like polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and flavones to induce rodent hepatic enzyme levels in our experimental studies. From these animals we make S9 or microsomes to activate HAs for mutagenicity testing. Now, because of the sensitivity of LC/MS/MS techniques, we can study various food ingredients, such as those found in broccoli, for their ability to change the human pharmacokinetics of the HAs in different individuals. Possibly, eating cruciferous vegetables with meat could lower risk, but more needs to be done to understand these interactions fully. Finally, the cell proliferation activity of HAs seems enhanced with other dietary exposures, such as specific herbal teas. Specifically, these combinations might be avoided in women susceptible to breast cancer or those trying to prevent re-occurrence of this disease. Understanding complex dietary exposures and competing risks should be studied in cell culture and rodents, but the ultimate understanding of these risks will only happen when we study humans individually and in large populations.

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PhIP metabolites in human urine after consumption of well-cooked chicken

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Abstract

We devised an assay to quantify the metabolites of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in human urine following a single exposure to well-cooked meat. Our method uses LC/MS/MS to detect four metabolites and four deuterated internal standard peaks in a single chromatographic run. N^2-OH–PhIP–N^2-glucuronide was the most abundant urinary metabolite excreted by the 12 individuals who participated in our study. N^2-PhIP glucuronide was the second most abundant metabolite for 8 of the 12 volunteers. The stability of PhIP metabolism over time was studied in three of the volunteers who repeated the assay eight times over a 2.5 year-period. PhIP metabolite excretion varied in each subject over time, although the rate of excretion was more constant. Our results suggest that quantifying PhIP metabolites should make future studies of individual susceptibility and dietary interventions possible.

Keywords: Food analysis; Metabolism; 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; Heterocyclic aromatic amine

1. Introduction

Potent genotoxic carcinogens of the heterocyclic amine (HA) class of compounds are produced in meat during cooking at high temperatures. The demonstrated mutagenicity of these compounds in bacteria [1], cells in culture [2,3] and mice [4], support the many studies of carcinogenicity in mice [5] and rats [6,7]. Mechanistic data show that, even at low doses, HAs form DNA adducts in rodents [8,9] and humans [10]. Of the 14 mutagens identified from cooked meat, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most mass abundant [11].

Humans are exposed to PhIP through the consumption of various cooked muscle meats, notably beef, pork and chicken [12–15]. The amount of PhIP that an individual is exposed to is related to food preparation methods [16–18], and the frequency of consumption. The presence of PhIP in restaurant and home-cooked meats has been documented, suggesting that humans may be exposed to PhIP in the range of 0.1–200 ng/g by consuming common foods [19–21]. These consumption levels may result in possible exposure doses in the milligram range for an individual.

The impact of heterocyclic amine exposure on human health is not clear, and its contribution to human cancer is a current subject of debate. Several epidemiological studies reported a positive correlation between the consumption of well-done meat and cancer risk [22–24]. In 1998, Zheng et al. [25] described a significant dose-dependence between meat preparation and breast cancer risk; women who preferred well-done hamburger, steak and bacon had a 4.6-fold greater risk of breast cancer than did women who preferred meats cooked "rare" or "medium". A recent case–control study of women in Shanghai, China showed a positive association of breast cancer risk and red-meat intake, especially well-done meat, which was more pronounced among women with a high body mass index [26]. Several studies reported an increased risk of colorectal adenomas and lung cancer with well-done and/or fried meat consumption [27–29]. African American males, who are at increased risk for prostate cancer, consume 2 to 3 times more PhIP than age-matched white males [30]. Two recent studies investigated the effect of *N*-acetyltransferase polymorphisms and cooked meat consumption on prostate cancer risk. Hein et al. [31] found that a particular subset of NAT2 acetylator genotypes were at increased risk for prostate cancer. In

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contrast, the study of Barrett et al. [32] provided no support for the hypothesis that fast NAT2 acetylators are at increased risk of colon cancer, even if exposed to high levels of HAs from well-cooked meats. Another study, performed in New Zealand, reported equivocal associations for well-done meat and prostate cancer [33]. Negative associations with cooked meat consumption have been reported with breast, colon, and rectal cancer [34–37].

PhIP is a procarcinogen that must be metabolically activated in order to damage DNA [38,39]. During Phase I metabolism PhIP is oxidized to the hydroxylated intermediates 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine (*N*-OH–PhIP) or 2-amino-1-methyl-6-(4′-hydroxy) phenylimidazo[4,5-*b*]pyridine (4′-OH–PhIP). Phase II metabolizing enzymes, primarily the acetyltransferases or sulfotransferases, then further convert *N*-OH–PhIP to a biologically active form that has been shown to bind DNA and cellular proteins [40–43]. Detoxification primarily involves glucuronidation. *N*-hydroxy–PhIP can form stable glucuronide conjugates at the N^2 and *N3* positions that can be excreted or transported to extrahepatic tissue for further metabolism [44,45]. 4′-Hydroxy–PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted [46,47]. In addition, the parent compound can be directly glucuronidated at the N^2 and *N3* positions. These glucuronides are not reactive and this reaction is believed to be a detoxification pathway [45,48]. Fig. 1 describes the formation of the four major human PhIP metabolites.

Human PhIP metabolism has been most intensively studied using hepatic microsomes or cells in culture. A recent study comparing PhIP metabolism in human and rat hepatocytes showed that the major human biotransformation pathway of PhIP was cytochrome P4501A2 (CYP1A2)-mediated N-oxidation followed by glucuronidation at N^2 and *N3* positions of PhIP [49]. In contrast, rat hepatocytes transformed PhIP to 4′-OH–PhIP as the primary product. Glucuronide and sulfate conjugates of 4′-OH PhIP were detected in human hepatocytes, but as relatively minor products [49]. Extrahepatic metabolism of PhIP has been demonstrated in breast, prostate, and colon. Studies have shown that human mammary cells have the capacity to metabolize the parent compound PhIP as well as the hydroxylated intermediates [50–52]. PhIP is glucuronidated by UGT1A1 in the human colon carcinoma cell line Caco2 [53] and human prostate cells have also been shown to metabolize PhIP ([54,55], Kulp, personal observations). The metabolic pathways and the metabolites produced during

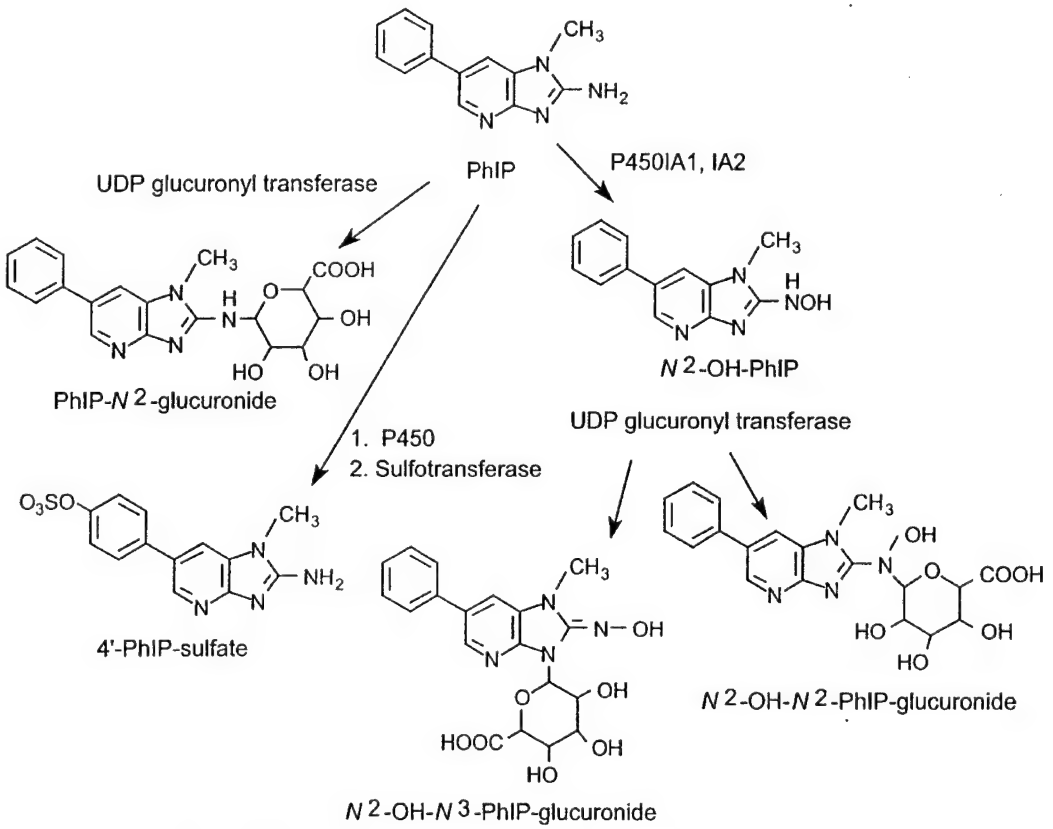

Fig. 1. Formation pathway for the major metabolites of PhIP found in human urine.

PhIP bioactivation in these target organs have not been fully determined.

Other studies of human PhIP metabolic pathways have been done in healthy volunteers by quantifying urinary metabolites. Pioneering work examined the relationship of urinary excretion of the unmetabolized parent compound and the dose received in well-done hamburgers [56,57]. PhIP and PhIP conjugates have been quantified in human urine using acid- or alkali-hydrolysis. These investigations demonstrate PhIP bioavailability, time course of excretion and the correlation between meat consumption and urinary metabolites, but do not give information about specific metabolic pathways [58–62]. Identification of human PhIP metabolites was determined in studies that investigated PhIP metabolism following administration of [^{14}C]-labeled PhIP to patients undergoing cancer surgery [63–65]. In these studies, body fluids and tissues were examined using accelerator mass spectrometry to investigate PhIP metabolic pathways. In 2002, Stillwell et al. [66] correlated the excretion of N^2–(β-1-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, measured as the deaminated product 2-OH PhIP, to CYP1A2 and NAT2 activity in 66 healthy subjects.

There have been four major PhIP metabolites identified in human urine: N^2-OH–PhIP–N^2-glucuronide, PhIP–N^2-glucuronide, PhIP–4′-sulfate, and N^2-OH–PhIP–*N3*-glucuronide [64]. Recently, we described a solid-phase extraction LC/MS/MS method for quantifying these four metabolites in human urine, following a meal of well-cooked chicken. We applied this method to characterize PhIP metabolism in healthy individuals receiving a known dose of naturally-produced PhIP [67,68]. We have also extended that method to examine the interactions of potentially preventive foods [69]. In the current study, we describe PhIP metabolism of 12 male volunteers, three of whom collected urine at 4 month intervals during a more than 2 years time span.

2. Material and methods

2.1. Study design

The study protocol was reviewed and approved by the Institutional Review Board for Human Research at Lawrence Livermore National Laboratory. Informed consent was obtained from each subject prior to beginning the study. The individuals participating were recruited from the local workforce, were all male, in good health, non-smokers, and of normal weight.

2.2. Meat preparation and controlled dietary period

Meat preparation conditions have been described previously [67]. Briefly, boneless, skinless chicken breasts were cut into approximately 2.5 cm pieces and fried in a non-stick coated pan, for 35–40 min. A representative chicken sample was removed for heterocyclic amine analysis. HA analysis was performed according to previously published methods [19]. The study subjects were provided with 150 g chicken with other non-meat foods and beverages. Total PhIP dose depended on the exact cooking time and was different for each batch of chicken cooked. The PhIP content in the various batches ranged from 61 to 131 ppb, providing doses of 9.2–19.6 μg PhIP in 150 g of cooked chicken. The PhIP dose was known for each subject. Two of the subjects repeated the assay eight times over the course of 2.5 years. A third subject repeated the assay seven times in the same time period.

Subjects were asked to abstain from meat consumption for 24 h prior to eating the well-done chicken breast. There were no other dietary restrictions. Control urine was collected before eating the chicken and for 24 h after in 6 h increments. Samples were coded, the volume recorded and stored frozen at −20 °C until analysis.

2.3. Extraction of PhIP metabolites

Urine samples (5 ml) were spiked with internal standard of urine (100 μl) from a rat dosed with pentadeutero-PhIP [70] (1 mg per day) generating the four different PhIP metabolites we detect in human urines. Samples were first applied to a pre-conditioned 60 mg Strata™ X SPE column (Phenomonex, Torrance, CA). Metabolites were eluted with 5 ml methanol. The elution aliquot was evaporated to dryness under nitrogen and the metabolites were re-dissolved in 2.5 ml 0.01 M HCl. Proteins and high molecular weight contaminants were removed by filtering the solution through a Centricon® YM-3 centrifugal filter (Millipore Corp., Bedford, MA). The filtrate was applied to a pre-conditioned benzenesulfonic acid column (SCX, 500 mg, Varian Sample Preparation Products, Harbor City, CA) and the column washed with 3 ml of 10% (v/v) methanol/0.01 M HCl. The metabolites were eluted onto a coupled C18 column (Bakerbond spe®, 1000 mg, J.T. Baker, Phillipsburg, NJ) with 60 ml of 0.05 M ammonium acetate, pH 8. The C18 column was washed with 3 ml of 5% (v/v) methanol/H_2O and eluted from the C18 column with 5 ml of 60% (v/v) methanol/H_2O. The metabolites were dried under nitrogen and 1 ml urine equivalent was injected into the LC/MS/MS in a volume of 20 μl.

Chromatography was done on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a YMC ODS-A column (3.0 mm × 250 mm). Metabolites were eluted at a flow rate of 200 μl/min using a mobile phase of A (water:methanol:acetic acid, 97:2:1) and 25% B (methanol:water:acetic acid, 95:4:1) with a linear gradient to 100% B at 20 min and held for 5 min.

Analytes were detected with a mass spectrometer (model LCQ, Finnigan, San Jose, CA) in the MS/MS positive ion mode using an electrospray interface. A capillary temperature of 240 °C, a source voltage of 4.5 kV, a sheath gas of 70

units and 5% auxiliary gas were used. An ion trap injection time of 1000 ms and one microscan were used.

Alternating scans were used to isolate $[M + H]^+$ ions at mass 417, 401, and 321 for natural PhIP metabolites, and 422, 406, and 326, for the pentadeutero-labeled internal standard metabolites. Collision energy was 25%. Daughter ions were detected at appropriate masses: 241 $[M + H$–glucuronic acid$]^+$ and 225 $[M + H$–glucuronic acid–$OH]^+$ from 417 for the *N*-hydroxy-N^2 and *N3* glucuronide, respectively, 225 $[M + H$–glucuronic acid$]^+$ from 401 for the PhIP N^2 glucuronide, 241 $[M + H–SO_3]^+$ from 321 for PhIP–4′-sulfate. Ion fragments detected for the deuterated internal standards were 5 mass units greater than the natural PhIP metabolite fragments.

2.4. Sample analysis and statistics

The overall recovery of the metabolites was determined by spiking each urine sample with known amounts of deuterium-labeled metabolites obtained from the rat urine. Final metabolite amounts were adjusted for losses based on the recovery of the internal standards. Each urine sample was analyzed at least twice. Total metabolite concentrations excreted in each time period were calculated by multiplying by the urine volume. Peak areas were converted to masses based on a response factor for PhIP and then normalized to percent of the original PhIP dose consumed in the chicken. Excretion rate was calculated by summing each of the four metabolites and calculating the percent of the total metabolites that were excreted in each time period. Spearman rank-correlation tests were used to determine the association between the excretion level of the N^2-OH–PhIP–N^2-glucuronide excreted and the ingested dose of PhIP. Thirty-three data pairs were used in the analysis; data from all 12 subjects as well as each individual subject's repetitions of the assay. Subjects were divided into "fast" and "slow" excretion groups based on a comparison of the amount of metabolite excreted in the 0–6 and 6–12 h time intervals. Subjects that excreted more metabolite in the 0–6 h interval were considered "fast", those that excreted more in the 6–12 h time interval were considered "slow". Average metabolites excreted by the two groups were compared using the Student's *t*-test.

3. Results

3.1. Human PhIP metabolite excretion after a meal of well-cooked chicken

Our method using LC/MS/MS detects peaks for the four identified human PhIP metabolites as well as four deuterated internal standard peaks in a single chromatographic run. Fig. 2 shows a set of mass chromatograms for a typical sample of the equivalent of 1 ml of urine injected. For increased sensitivity, the data acquisition was made over three segments, isolating mass 321 for 14 min, masses 417, 401, and 422 for 7 min, and mass 417 only for the final 5.5 min. Since other ion peaks are often present in the chromatograms that do not represent one of the four identified PhIP metabolites (Fig. 2), expected peak retention times and peak widths are compared to reference samples to confirm the identity of the PhIP metabolites. N^2-OH–PhIP–N^2-glucuronide and its deuterium-labeled analog are detected as broader HPLC peaks that fragment into two daughter ions. The sum of these two peak areas is used for quantitation (Fig. 2). The N^2-OH–PhIP–*N3*-glucuronide is separated in time from the N^2-OH–PhIP–N^2-glucuronide and fragments to mass 225 only (Fig. 2). HPLC column lifetime is a problem with these samples. We slurry-pack our own columns with 10 μm particle size resin and replace the column after 24 injections to obtain the best results for routine samples.

Control urine samples were collected before the well-done chicken was consumed, during the period that the volunteers abstained from eating cooked meat. No PhIP metabolite peaks were seen in the control samples from the 12 individuals. Total urine excreted after chicken consumption was collected for 24 h in 6 h increments. Metabolite values shown are corrected for the total volume of urine. Fig. 3 shows the percentage of the ingested PhIP dose recovered in the urine as PhIP metabolites for the 12 subjects. Recovered doses varied nine-fold despite the fact that all urine was collected and amounts were normalized to account for differences in PhIP dose. The total amounts of each of the four individual metabolites excreted during the 24 h collection period are also shown in Fig. 3 as variably shaded regions of the bars. N^2-OH–PhIP–N^2 glucuronide was the most abundant urinary metabolite in all individuals, comprising 44 (Subject K) to 80% (Subject F) of the total metabolite excreted. N^2-PhIP glucuronide was the second most abundant metabolite for 8 of the 12 volunteers and these two metabolites together account for 77–95% of the total metabolite excretion for these individuals. In three individuals (B, L, and M) N^2-OH–PhIP–*N3*-glucuronide was the second most abundant metabolite and in Subject H PhIP–4′-sulfate was second most abundant, comprising almost 30% of the total metabolite excreted.

Fig. 4 shows the rate of excretion of the PhIP metabolites in time periods of 0–6, 6–12, 12–18 and 18–24 h. Subject L did not provide a sample for the 12–18 h period. Subjects F and G provided sample for the 18–24 h time period, but no metabolites were detected in these samples. In all of the subjects, the majority of the metabolites were excreted in the first 12 h (61–92%). The individuals showed variation in the time of metabolite excretion. Six of the subjects (A, E, G, K, L and M) excreted more than 45% of the total metabolite in the 6–12 h time period. The other six individuals excreted 34–50% of the total metabolite in the 0–6 h time period. Seventy-six to 100% of the dose was excreted in the first 18 h after consuming the cooked chicken meal.

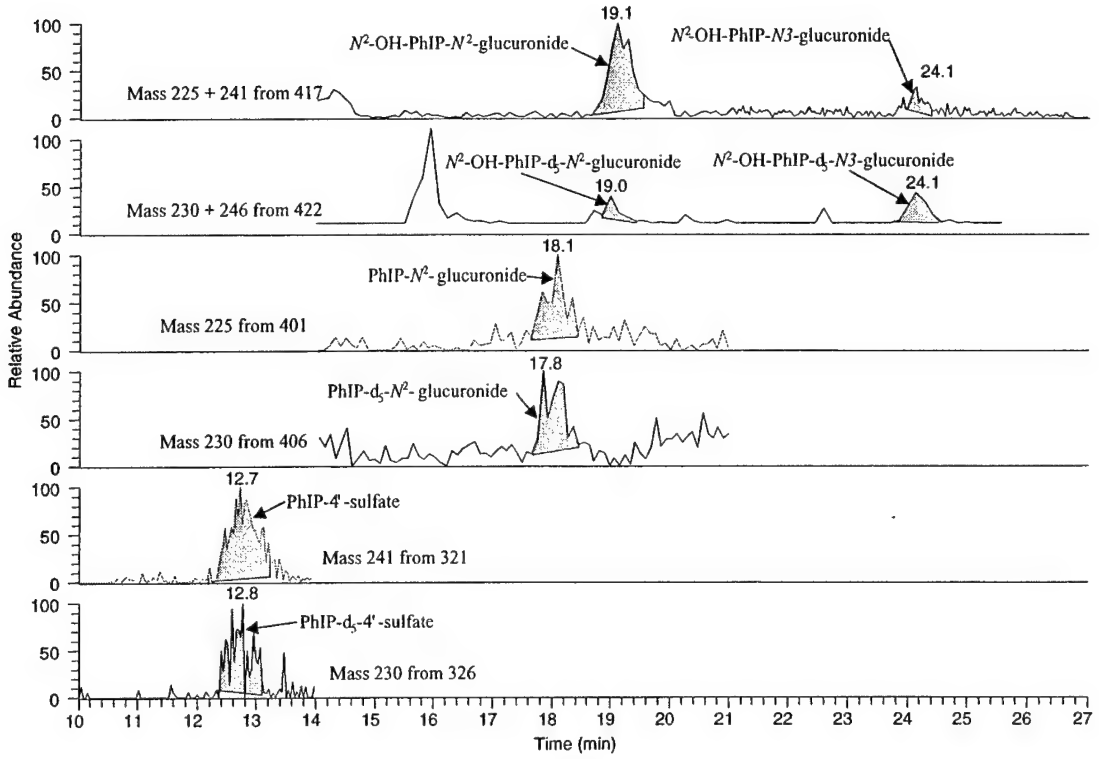

Fig. 2. Ion plots of PhIP metabolites and the pentadeutero-PhIP (PhIP-d_5) metabolite internal standards from the injection of the equivalent 1 ml of urine. See Section 2 for LC/MS/MS conditions.

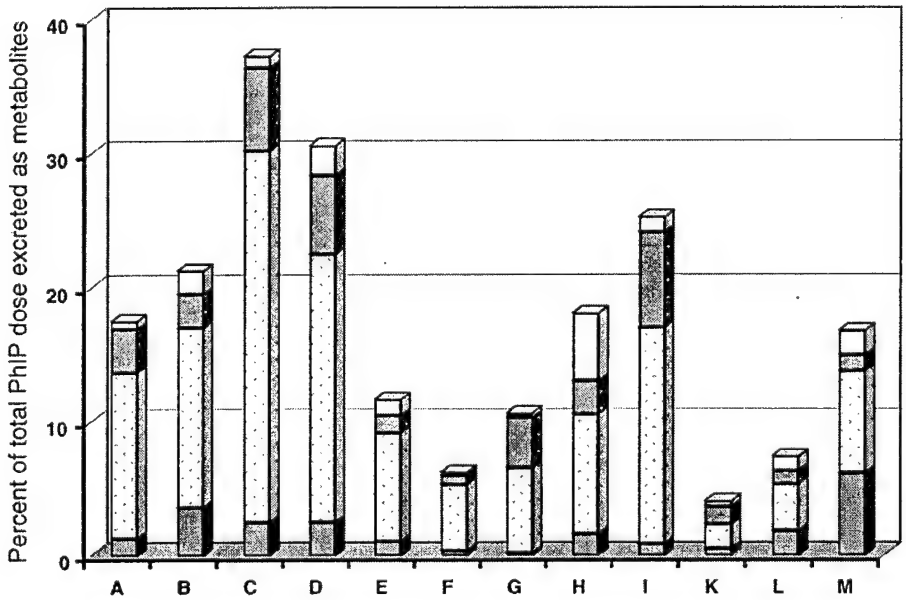

Fig. 3. Total 24 h excretion of urinary PhIP metabolites for 12 subjects. Total excretion of each metabolite during the 24 h time period was calculated and expressed as percent of the PhIP dose ingested. (▒) N^2-OH–PhIP–$N3$-glucuronide; (▒) N^2-OH–PhIP–N^2-glucuronide; (▓) PhIP–N^2-glucuronide; (▭) PhIP–4′-sulfate.

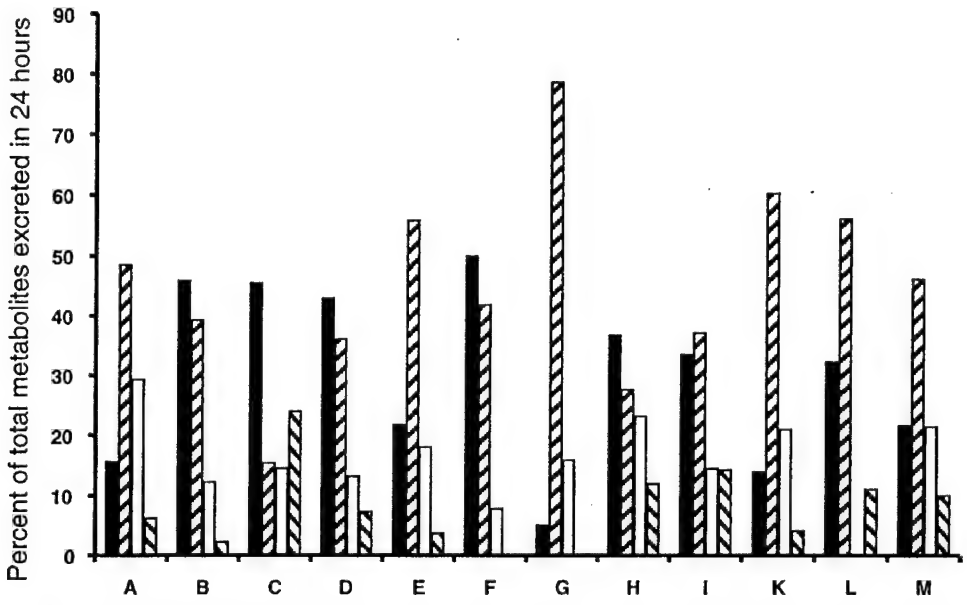

Fig. 4. Rate of excretion of four PhIP metabolites for 12 subjects. Total urinary metabolites recovered during the 24 h after dosing were quantified. Data represent the percentage of the total metabolites excreted during the designated time period. Time increments shown are: (■) 0–6; (▨) 6–12; (□) 12–18; (▧) 18–24 h.

3.2. Correlation of metabolites excreted to PhIP dose ingested

A weak association was observed (Fig. 5) between the amount of PhIP ingested and the total amount of N^2-OH–PhIP–N^2 glucuronide excreted in the 24 h urine ($r_s = 0.29$, $P < 0.1$). We also compared the total amount of PhIP ingested to the sum of all of the metabolites excreted, but this did not improve the correlation.

3.3. Comparing "fast" and "slow" excretion groups

The subjects in the study were divided into "fast" and "slow" excretion groups based on the amount of metabolites

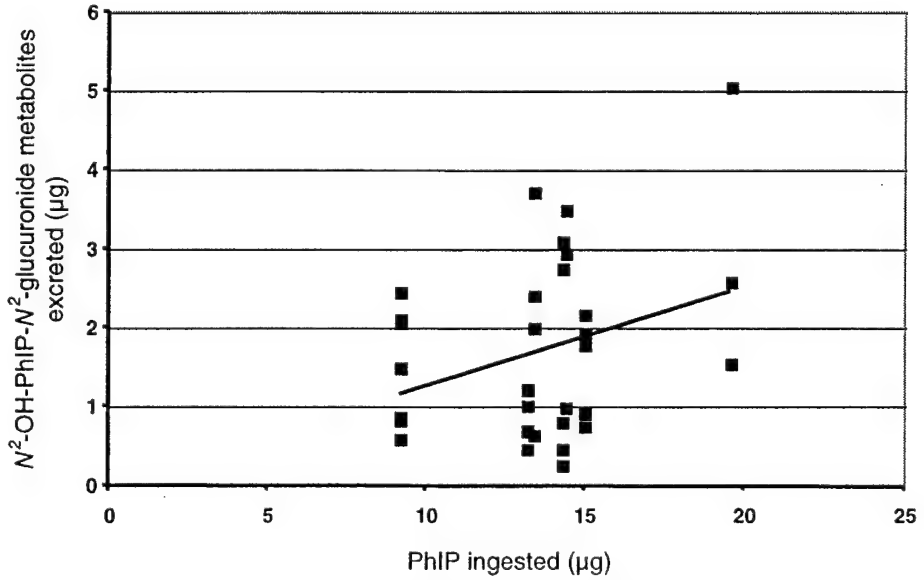

Fig. 5. Linear regression analysis of total N^2-OH–PhIP–N^2-glucuronide excreted 0–24 h after chicken consumption as a function of the amount of the PhIP consumed for each individual.

Table 1
Average metabolite excretion of "fast" and "slow" excretion groups

	N^2-OH–PhIP–*N3*-glucuronide	N^2-OH–PhIP–N^2-glucuronide	PhIP–N^2-glucuronide	PhIP–4′-sulfate	Total
Fast	3.2 ± 3.2	20.6 ± 15.1	5.5 ± 5.1	2.5 ± 1.9	31.8 ± 19.6
Slow	1.6 ± 1.4	10.4 ± 6.9[a]	2.7 ± 1.9	0.9 ± 0.6[a]	15.6 ± 8.2[a]

Data are means ± standard deviation.
[a] Fast significantly different than slow ($P < 0.05$).

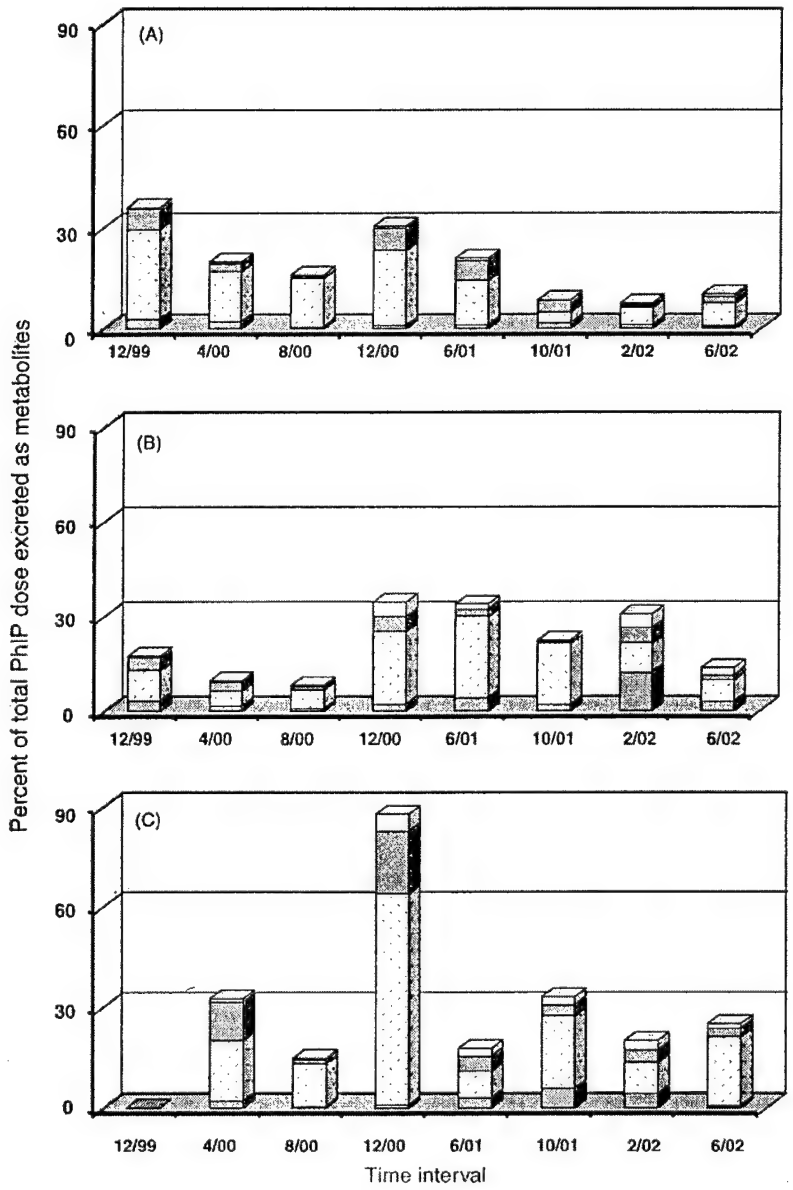

Fig. 6. Total 24 h excretion of urinary PhIP metabolites for three subjects at several different times. Total excretion of each metabolite during the 24 h time period was calculated and expressed as percent of the PhIP dose ingested. (A) Subject A; (B) Subject B; (C) Subject C. (▨) N^2-OH–PhIP–*N3*-glucuronide; (▧) N^2-OH–PhIP–N^2-glucuronide; (▩) PhIP–N^2-glucuronide; (▭) PhIP–4′-sulfate.

excreted in the 0–6 h time period. Subjects were considered "fast" excretors if the ratio of the metabolites excreted in the 0–6 h time interval to the 6–12 h time interval was greater than or equal to 1. "Slow" excretors were defined as a ratio less than 1. The average metabolite excretion for each group is presented in Table 1. Subjects considered "fast" excreted significantly more N^2-OH–PhIP–N^2 glucuronide, 4′-PhIP sulfate and total metabolites than the subjects considered "slow" ($P < 0.05$).

3.4. Human PhIP metabolism in three individuals over time

To determine individual changes in PhIP metabolism over time, we measured PhIP metabolite excretion in 3 subjects repeatedly over a 2.5 year-period (Figs. 6 and 7). The assay was repeated at approximately 4 month intervals. Subject C did not participate in the assay in December 1999. As seen in Fig. 6, the amount of PhIP metabolites excreted, expressed

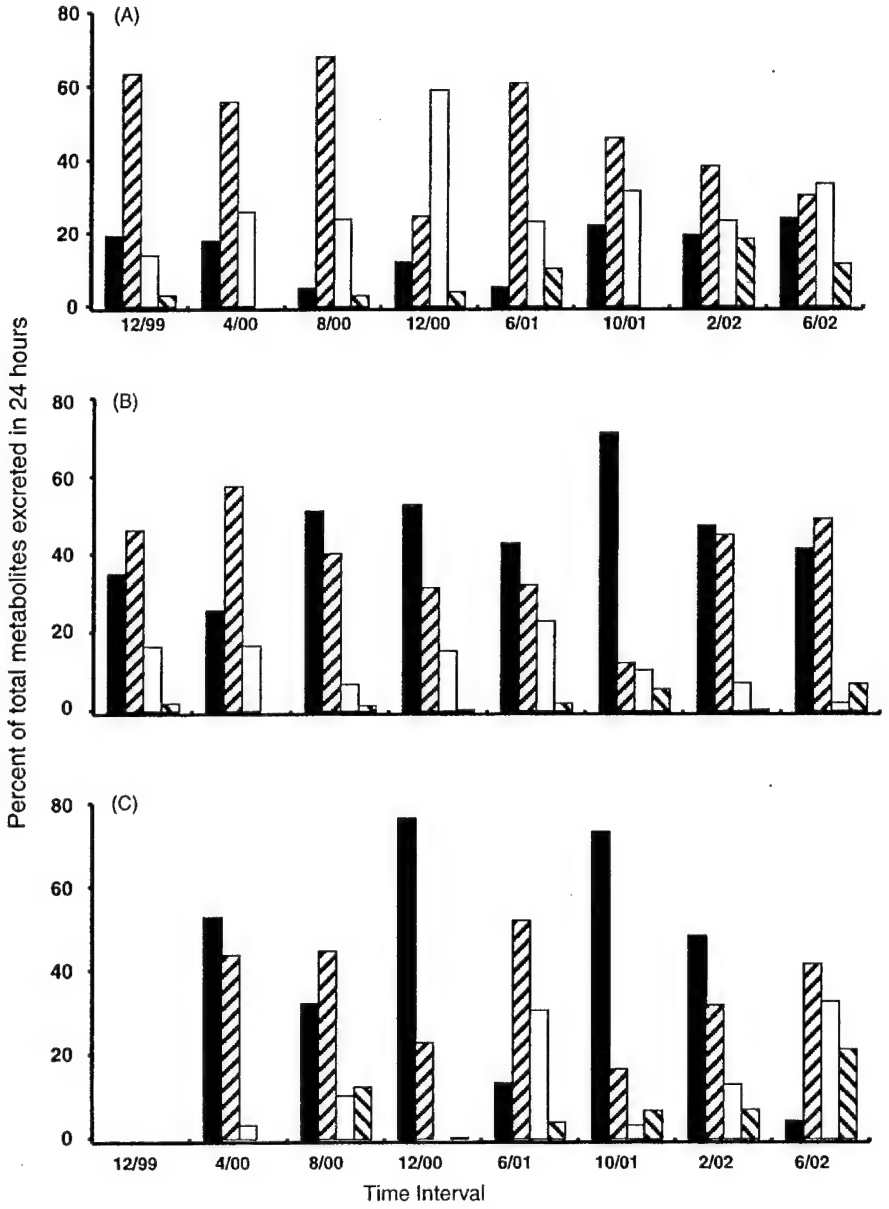

Fig. 7. Rate of excretion of four PhIP metabolites for 12 subjects. Total urinary metabolites recovered during the 24 h after dosing were quantified. Data represent the percentage of the total metabolites excreted during the designated time period. Time increments shown are: (■) 0–6; (▨) 6–12; (□) 12–18; (▧) 18–24 h.

as percent of the PhIP dose ingested, is not constant in these individuals over time. Considerable variation exists not only in the amount of each individual metabolite excreted (shown as variably shaded regions within the bar) but in the total amount of the PhIP dose excreted as well. In contrast, the rate of metabolite excreted is more constant (Fig. 7). For Subject A, the larger fraction of the metabolites excreted was always in the later time intervals; 6–12 h or 12–18 h. Subject B, on the other hand, tended to excrete metabolites more quickly; in five of the eight trials the largest fraction of the metabolites were excreted in the 0–6 h time interval. Subject C, similarly to Subject B, excreted the largest fraction of the metabolites in the 0–6 time interval in four out of seven trials. Both B and C excreted almost all of the metabolites in first 12 h after consuming chicken (an average of 80% for both subjects over all time intervals), whereas Subject A excreted an average of 64% of the metabolites in the first 12 h.

4. Discussion

This study reports the variation in PhIP metabolism among twelve healthy human subjects who have been fed a single meal containing well-cooked chicken. Both the amounts of chicken consumed by our volunteers and the PhIP levels were comparable to consumption levels possible in households or restaurants [71].

In the present study, we found that the amount of metabolites excreted in the 0–24 h urine represented 17 ± 10% of the ingested PhIP. In a previous study of normal females, we reported a similar average of 21.5% of the PhIP dose recovered in the urine [67]. Strickland et al. [60] reported that 16.6% of the ingested PhIP could be quantified in the 0–12 h acid-hydrolyzed urine of their population and Stillwell et al. [66] reported the recovery of N^2-OH–PhIP–N^2-glucuronide (measured as 2-OH–PhIP) as an average of 24.6%. These studies all confirm that PhIP present in the meat matrix is not completely bioavailable. In an earlier study of hospitalized elderly cancer patients given PhIP in a gelatin capsule, 90% of the ingested dose was recovered in the urine for two of the three subjects [64]. This indicates that PhIP provided in capsule form is more bioavailable than PhIP ingested in meat. We are currently investigating the bioaccessibility of PhIP from cooked meat using an in vitro digestion model. In that study we show that release of PhIP from the meat matrix was dependent upon pancreatic enzyme concentration and meat doneness [72]. We are also investigating the impact that other foods in the GI tract may have on PhIP bioaccessibility. Other factors such as transport across the intestinal cell monolayer and individual differences in metabolic pathway capacities may also ultimately affect how much of the ingested PhIP dose is recoverable in the urine.

The kinetics of PhIP metabolite excretion in our study are similar to those seen previously for humans [56,64]. Our results demonstrate that excretion times vary among the volunteers but that most of the dose (76–100%) is excreted in the first 18 h. This suggests that these metabolites are suitable for investigating individual variation in rates and ratios of PhIP metabolism. Further, these metabolite measurements may be used as biomarkers of recent exposure, but are not suitable for long-term exposure estimates.

The detection of individual metabolites also confirms our earlier findings [64]. The ratio of the individual metabolites varied among our 12 individuals, although N^2-OH–PhIP–N^2-glucuronide was always the most abundant. In our previous study of female volunteers, we also found N^2-OH–PhIP–N^2-glucuronide in the greatest amounts, although in the female volunteers PhIP–N^2-glucuronide was consistently the second most abundant. In the current study, we found that N^2-OH–PhIP–*N3*-glucuronide and PhIP–4′-sulfate, which were minor metabolites in the female population, contributed substantially to the total metabolite excretion. Other studies have investigated the effect of gender difference on PhIP dose–response relationships and excretion of 2-OH–PhIP and found no significant association [60,66]. However, neither of these studies identified and compared the excretion of the specific PhIP metabolites that are noticeably different in our studies.

In 1997, Reistad et al. [57] found that 4′-OH–PhIP could be detected in cooked meat as well as in human urine. Although the same result has not been shown for cooked chicken, it is possible that the 4′-PhIP-sulfate detected in the urine may be formed from 4′-OH–PhIP found in the cooked meat, rather than a metabolite of the ingested parent compound.

Our results demonstrate only a weak association between metabolites excreted and PhIP dose ingested. Other studies of more individuals have reported much stronger correlations [60,66]. Analyzing the average metabolite excretion of the "fast" versus "slow" individuals demonstrated that volunteers who excreted metabolites more quickly excreted significantly more N^2-OH–PhIP–N^2-glucuronide, PhIP-4′-sulfate and total metabolites than the individuals who excreted more slowly. It is possible that individuals that excrete more slowly excrete less metabolite because (1) less compound is being absorbed or it is being absorbed more slowly, (2) more of the compound is being sequestered in the tissues, or (3) the compound is being processed by other, unidentified pathways.

However, due to the small size of our study population, it is impossible to attribute meaning or significance to any of these intriguing results. More work will need to be done in much larger populations to verify trends in gender differences or differences in excretion rate.

We repeatedly analyzed PhIP metabolism in the same three individuals over time to determine the consistency of metabolite excretion. We found that both the percent of the dose excreted in the urine as well as the amounts of each of the metabolites was highly variable in each individual. Although the rate of metabolite excretion appeared to be more constant over time (one of the individuals was

consistently slow, the other two were more often fast), the percentages of metabolites that were measured in each time interval also varied widely. Given the numerous reports of diet and lifestyle affecting metabolizing enzyme activity it is not surprising that there are metabolic variations in individuals eating a normal diet over time. Although these differences may make correlating PhIP metabolite excretion with genotype more difficult, it does suggest that it is possible to devise dietary intervention strategies to reduce the impact of PhIP exposure. Of the metabolites we detected, two appear to be part of the activation pathway for PhIP, N^2-OH–PhIP–N^2-glucuronide and N^2-OH–PhIP–*N3*-glucuronide [67]. It is likely that interventions that reduce the *N*-hydroxylation of PhIP or increase the direct glucuronidation of PhIP are desirable. We are currently investigating the effect of potentially chemopreventive foods on PhIP metabolism in small populations.

Altering the metabolism of PhIP to prevent formation of biologically active species may reduce individual susceptibility and prevent the occurrence of cancers in target tissues. Our results suggest that quantifying PhIP metabolites should make studies of individual susceptibility and dietary interventions possible in the future.

Acknowledgements

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Factors affecting human heterocyclic amine intake and the metabolism of PhIP

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Abstract

We are working to understand possible human health effects from exposure to heterocyclic amines that are formed in meat during cooking. Laboratory-cooked beef, pork, and chicken are capable of producing tens of nanograms of MeIQx, IFP, and PhIP per gram of meat and smaller amounts of other heteroyclic amines. Well-done restaurant-cooked beef, pork, and chicken may contain PhIP and IFP at concentrations as high as tens of nanograms per gram and MeIQx at levels up to 3 ng/g. Although well-done chicken breast prepared in the laboratory may contain large amounts of PhIP, a survey of flame-grilled meat samples cooked in private homes showed PhIP levels in beef steak and chicken breast are not significantly different ($P = 0.36$). The extremely high PhIP levels reported in some studies of grilled chicken are not seen in home-cooked samples.

Many studies suggest individuals may have varying susceptibility to carcinogens and that diet may influence metabolism, thus affecting cancer susceptibility. To understand the human metabolism of PhIP, we examined urinary metabolites of PhIP in volunteers following a single well-done meat exposure. Using solid-phase extraction and LC/MS/MS, we quantified four major PhIP metabolites in human urine. In addition to investigating individual variation, we examined the interaction of PhIP with a potentially chemopreventive food. In a preliminary study of the effect of broccoli on PhIP metabolism, we fed chicken to six volunteers before and after eating steamed broccoli daily for 3 days. Preliminary results suggest that broccoli, which contains isothiocyanates shown to induce Phases I and II metabolism in vitro, may affect both the rate of metabolite excretion and the metabolic products of a dietary carcinogen. This newly developed methodology will allow us to assess prevention strategies that reduce the possible risks associated with PhIP exposure.

Keywords: PhIP; MeIQx; IFP; Heterocyclic amine; Food mutagen

1. Dietary intake and heterocyclic amine carcinogens

Human epidemiologic and animal studies have shown that diet has a role in the etiology of human cancer. Diet is one aspect of an individual's lifestyle that may be practically modified. Therefore, it is important to quantify dietary exposures to understand an individual's risk for cancer and to identify habits or practices that increase or decrease an individual's risk. Although complex, the interactions between the myriad different components in the whole diet may be a critical factor in determining the likelihood of cancer initiation.

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There is general consensus that potent genotoxic carcinogens are produced in meat during cooking at high temperatures. The demonstrated mutagenicity of these compounds in bacteria [1], cells in culture [2,3] and mice [4,5], support the many studies of carcinogenicity in mice and rats [1,6]. Mechanistic data show DNA adducts in rodents and humans consuming these compounds at low doses [7].

Although, the role of heterocyclic amines in cancer initiation has been well-established in animals, much less is known about the effect of heterocyclic amine exposure on tumor development in humans. The presence of heterocyclic amines in commonly consumed commercially cooked meats has been well-documented [8,9] and risk assessments made using the available data [10–12]. Depending on individual dietary and cooking preferences, human intake of heterocyclic amines may range from nanograms to micrograms per day.

2. Comparison of heterocyclic amines with other aromatic amines and the relationship to human cancer

The precedent for aromatic amines causing human cancer comes from occupational exposures in the chemical industry. In one case, all 15 workers distilling 2-naphthylamine developed bladder cancer [13]. Ward et al. showed a relative risk of 27 for bladder cancer in workers occupationally exposed to *ortho*-toluidine and aniline for greater than 10 years [14].

So, for heterocyclic amines in foods, bladder cancer might be the logical endpoint. Esophageal tumors, in addition to bladder tumors, were seen in two studies [15,16], but not in a third [17], suggesting, at least, that other tumor sites may be relevant for aromatic amine exposure.

Are the low amounts in present in some cooked meats safe because of a threshold needed to induce tumors? The doses are not known for the occupational exposures cited above, so neither the dose needed to cause the human bladder tumors, nor the difference between the occupational dose and the dietary human heterocyclic amine dose can be determined.

Gender differences are known in human bladder cancer, with males being more sensitive [18]. For well-done meat and colorectal cancer, there was a non-significant two-fold increase in males, but not in females [19]. Are mixed gender studies of aromatic amine carcinogenesis confounded? Gender differences are just beginning to be investigated in laboratory studies and need further investigation.

Recently epidemiologists have begun investigating possible links between well-done meat consumption and cancer risk. Several epidemiology studies have reported an increased risk of cancer associated with subject groups that prefer well-done meat. In 1998, Zheng et al. described a significant dose-response relationship between doneness levels of meat and breast cancer risk; women who preferred well-done hamburger, steak and bacon had a 4.6-fold greater risk of breast cancer than did women who preferred meats cooked "rare" or "medium" [20]. Other studies reported an increased risk of colorectal adenomas with increased well-done meat consumption [21,22]. Lung cancer risk has also been related to the consumption of fried, well-done meat [23]. Other studies, however, have shown either equivocal associations with well-done meat and cancers of the prostate gland [24] or negative associations with cancers of the breast [25,26], colon or rectum [11].

In all of these studies, heterocyclic amine exposure levels are based upon answers to dietary questionnaires. However, the formation of heterocyclic amines in foods depends on many cooking variables, and dietary surveys give varying estimates of heterocyclic amine dose that may or may not reflect actual exposures.

3. Prediction of heterocyclic amine intakes from dietary questionnaires

Precisely quantifying the dietary dose of heterocyclic amines in the population and individuals is essential for risk determination. The most common practice in epidemiology is to establish dietary exposure through questionnaires. These questionnaires typically use subject recall to determine the amount of meat consumed, the preparation method, and the doneness of the meat, with photographs sometimes used to estimate doneness. These parameters are all then linked to databases of heterocyclic amine content. Although these estimates of intake have been

used with the belief that they are highly precise [21], none of the current epidemiological studies are able to estimate accurate exposures, because no biomarkers of dose have been used to validate the questionnaires in any of these studies.

White meat (fish and chicken breast) has been frequently identified as a confounding factor in studies of heterocyclic amine exposure and cancer relationship. Consumption of white meats is generally associated with lower cancer rates, yet these meats have been attributed with heterocyclic amine exposures that are greater than red meats [27–29]. In an early study of laboratory-cooked chicken [30], high levels of PhIP were found in chicken samples that have not been shown to be typical in even the most well-done meat diet in our recent work. Cooking method and the interpretation of meat doneness are responsible for a great deal of variation in heterocyclic amine amounts, especially for PhIP in chicken.

For example, marinating meat is a preparation method generally not accounted for in dietary questionnaires for heterocyclic amine exposure assessment. Fig. 1 shows the formation of PhIP in chicken breast meat as a function of weight loss during cooking. Analysis was performed on meats grilled, fried, or broiled in our laboratory or on meat samples that had been sent to us previously cooked [30]. Only when chicken breast is cooked to extreme dryness (weight losses of 40% or more), do PhIP levels increase to the very high levels occasionally found. Because weight loss and the perceived dryness of the food is used as a measure of cooking doneness, it is apparent from Fig. 1 that determining when samples are "done" can have a great effect on PhIP levels. Also shown in Fig. 1 is the effect of marinating on PhIP formation. As we have described previously, marinating before grilling greatly reduces PhIP levels in chicken [31]. Notably, in samples cooked to the same degree of weight loss, PhIP levels are up to 10-fold less in the marinated samples. These results emphasize the extreme differences in PhIP levels that can occur as a result of different cooking methods.

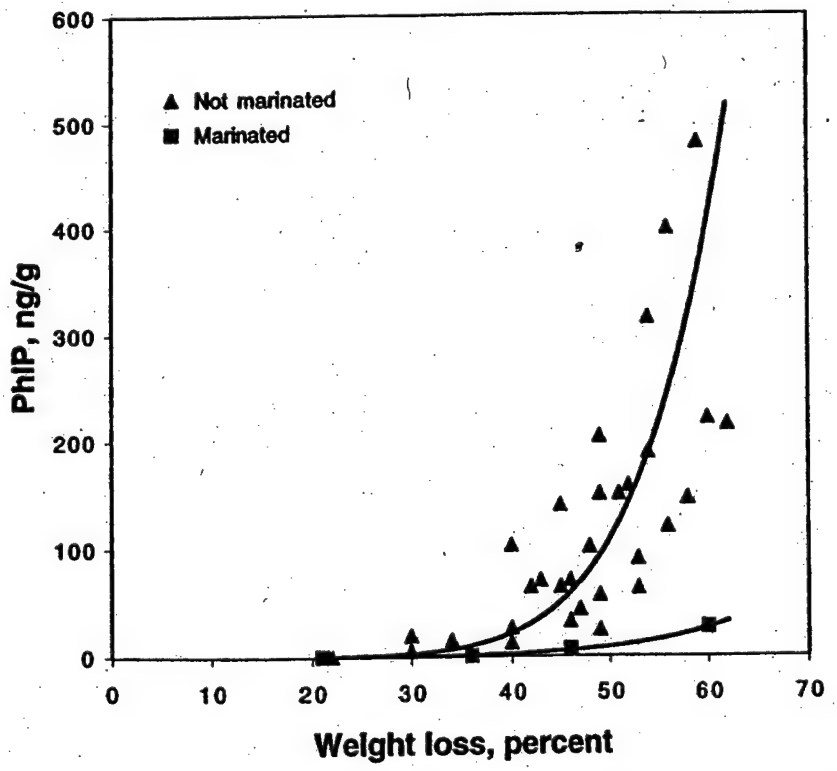

Fig. 1. The PhIP content and weight loss during grilling, frying or broiling of chicken breast. Samples marinated before cooking (squares) show low PhIP levels despite great weight loss during flame grilling.

Another uncertainty surrounds the heterocyclic amine databases used to construct exposure categories. Most epidemiologic studies of heterocyclic amines use relationships between heterocyclic amine concentrations and doneness level derived from laboratory cooking studies. However, heterocyclic amine levels in meats obtained from homes have varied considerably from the laboratory data. In a study of foods cooked under actual household conditions, grilled meat samples were obtained from households in the midwestern US. Samples were taken from volunteers responding in survey that they preferred their meat well-done or very well-done, leading us to expect high heterocyclic amine exposures in these households. Ninety-two samples of cooked meat, including 20 samples each of flame-grilled ground beef patties, pork or chicken parts and 32 samples of grilled steaks were obtained and analyzed by solid-phase extraction and photodiode-array HPLC using published methods [8].

MeIQx and PhIP values for the four different kinds of cooked meats and their averages are plotted in Fig. 2. Samples with no detectable amounts of PhIP and MeIQx were assigned a value representing half of the lowest level of detection: 0.02 ng/g for MeIQx, and 0.08 ng/g for PhIP. As expected for well-done meats, PhIP, on average, was found in greater amounts than MeIQx in each of the meat types. The biggest range of PhIP values was found in the chicken breast, undetectable levels to 48 ng/g, followed by the grilled steak and the beef patties. Pork had the smallest range of PhIP values (0–7 ng/g). The amount of MeIQx found in the samples ranged from 0 to 7 ng/g in grilled steak and chicken, 0 to 3 ng/g in grilled beef patties and 0 to 2 ng/g in pork. Surprisingly, in this collection of well-done or very well-done meats samples approximately 25% of the samples had undetectable levels of MeIQx or PhIP as shown in Table 1. Approximately 20% of the samples across all doneness categories had no detectable heterocyclic amines of any kind. Although chicken breast had some of the highest PhIP values, a comparison of PhIP levels in chicken breast and beef steak shows that the amounts of PhIP formed in the two meat types are not significantly different ($P = 0.36$) from each other.

The high variability observed in these home-cooked samples, especially for PhIP in very well-done chicken, may contribute to the contradiction of white-meat associated low cancer rates and high heterocyclic

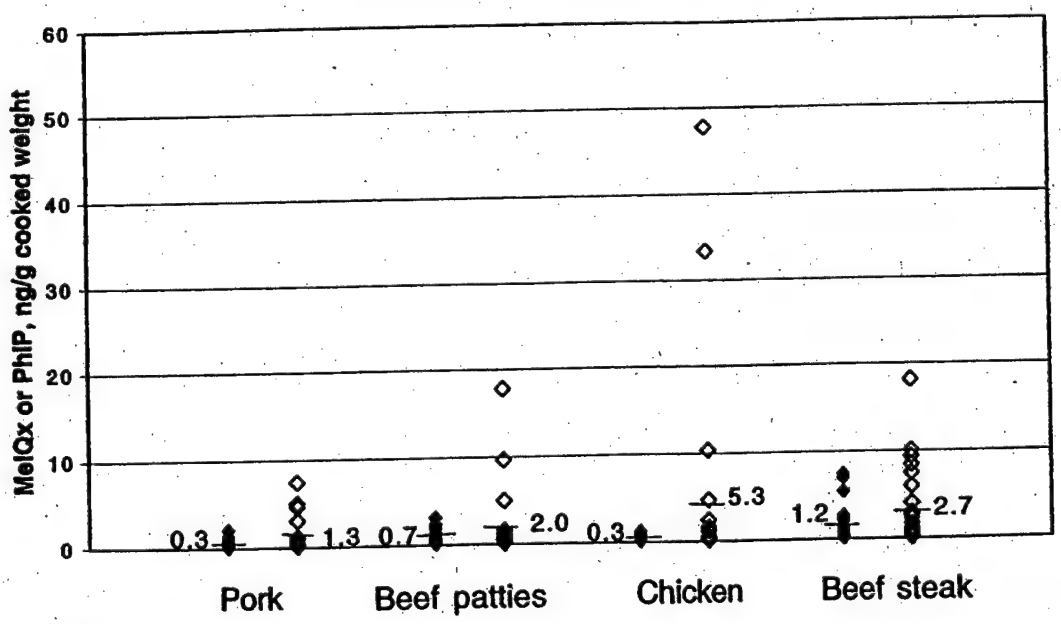

Fig. 2. Plot of MeIQx (filled symbols) and PhIP (open symbols) in grilled meat samples obtained from homes in the US whose occupants stated in a survey that they preferred their meat well-done or very well-done. Averages for each compound and meat type are shown; $n = 20$ samples for beef patties, pork, and chicken, $n = 32$ for steak.

Table 1
Percentage of samples with no detectable levels of PhIP, MeIQx or all heterocyclic amines obtained from homes specifying a preference for well-done or very well-done meats.

Meat	PhIP	MeIQx	All heterocyclic amines
Chicken	25% (5/20)	20% (4/20)	15% (3/20)
Beef steak	22% (7/32)	15% (5/32)	12.5% (4/32)
Pork	50% (10/20)	35% (7/20)	30% (6/20)
Beef patty	30% (6/20)	25% (5/20)	25% (5/20)

amine exposure. Using high heterocyclic amine values reported in an early study of laboratory-cooked chicken [30], Byron et al. concluded that chicken prepared by grilling, broiling, or pan-frying are the three foods that most reliably predict PhIP exposure [27]. However, based upon the results presented in Fig. 2, as well as analysis of meat cooked in restaurants [32], we believe that the levels of PhIP are similar in chicken and beef when the meat is cooked in typical households. In the same study by Byrne et al., broiled fish was identified as the fourth "predictor of PhIP exposure." In studies of fish cooked to the doneness usually eaten in the US or Sweden, there is little evidence in support of the conclusion that broiled fish contains more PhIP than beef steaks [33,34]. The research group that reported large amounts of PhIP in well-cooked salmon [35] found no PhIP in another grilled fish type in a follow-up study that compared laboratory grilled beef, pork (bacon), and fish [36]. Yet the latter study is not often considered when assessing dietary intake.

Based on these observations it is apparent that quantifying human heterocyclic amine exposure is not a simple task. Formation of heterocyclic amines in meat during cooking is highly dependent upon cooking method and doneness levels. Individual exposure depends upon meat consumption patterns. The compelling conclusion from these meat and cancer studies is that humans may be exposed to genotoxic carcinogens over a lifetime. Intake levels are low; still, one microgram of MeIQx (a 200 g steak with 5 ng/g) has 2.8×10^{15} molecules that can be absorbed, and then activated or detoxified through metabolism. Clearly, focusing on just doneness level simplifies efforts to estimate heterocyclic amine exposure but will overlook important variables such as cooking practices and meat type.

4. Human PhIP urinary metabolites as a measure of metabolism phenotype

The enzymes known to be involved in the metabolism of heterocyclic amines are found at a variety of levels and activities within the human population [37]. Variation in the expression of these enzymes suggests variation in the amounts of the activation compared to the detoxification intermediates produced. Changes in the activity of these enzymes can occur due to changes in lifestyle habits and diet. Altering the metabolism of heterocyclic amines by altering the activity of metabolizing enzymes may prevent formation of biologically active species and thus may prevent the occurrence of cancer.

One way of monitoring human metabolic activation/detoxification patterns and possibly identifying individuals that may be more or less at risk for cancer initiation is through measuring the excretion of heterocyclic amine metabolites in the urine. Identifying and quantifying metabolites produces a "snapshot" of recent exposure as well as a way to monitor changes in metabolic enzyme activity. We developed a method for quantifying PhIP metabolites in human urine following a single meal of well-done meat.

Pioneering work in in vivo human metabolism examined the relationship between urinary excretion of the unmetabolized parent compound and the dose received in well-done hamburgers [38,39]. Other studies demonstrated the presence of PhIP and PhIP conjugates in human urine, but in these studies the urine was first treated with acid to hydrolyze the Phase II metabolic conjugates to the parent amine. These investigations showed that PhIP is bioavailable in humans, but did not give information about specific metabolic pathways [40–42].

Most recently, specific results about the identity of human PhIP metabolites were obtained in studies that investigated human PhIP metabolism following administration of [^{14}C]-labeled PhIP to patients undergoing cancer surgery [43–45]. Surprisingly, the relative amounts of human urinary metabolites were unlike those of rodents and more like those of dogs [44]. These studies identified four major human PhIP metabolites: N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, PhIP-4′-sulfate, and N^2-OH-PhIP-N^3-glucuronide. Based on the metabolite identification, we developed a solid-phase extraction, LC/MS/MS

method that quantifies the four known PhIP metabolites in human urine, following a single meal of well-cooked chicken [46]. Chicken is used in this assay because we can produce PhIP in overcooked chicken without a concomitant amount of other known heterocyclic amines. Because the PhIP is formed naturally in the chicken at levels that represent possible dietary exposures, we can apply this method to characterize PhIP metabolism in normal, healthy volunteers.

To determine the feasibility of affecting PhIP metabolism by dietary supplementation with a putative chemopreventative food in humans, we investigated the effect of broccoli on PhIP metabolism by quantifying changes in PhIP urinary metabolites. The study protocol was reviewed and approved by the Institutional Review Board for Human Research at Lawrence Livermore National Laboratory, and informed consent was obtained from each subject prior to beginning the study. The individuals participating were recruited from the local workforce, were in good health, non-smokers and of normal weight. The meat was prepared by cutting boneless, skinless chicken breasts into approximately 2.5 cm pieces and pan-frying for 25–35 min. The chicken pieces generally lost 40% of their weight during cooking, and at the end of the cooking time the chicken appeared white with some browning. A representative sample was removed and analyzed for PhIP content using published methods [8]. Volunteers were provided with 150 g of chicken containing from 60–90 ppb PhIP for a total dose of 9–13 μg.

In this preliminary study, we fed six human male volunteers well-cooked chicken, collected urine, and measured a baseline urinary PhIP metabolite profile. We then gave the subjects one cup of steamed broccoli daily for 3 days. On day 4 we fed them chicken again and collected urine. Three of the individuals have repeated the procedure at 3-month intervals. Volunteers were asked to not eat grilled meat for 24 h prior to eating the chicken meal and to abstain from broccoli and related cruciferous vegetables for 3 days before the intervention. No further dietary restrictions were imposed. All subjects were provided with other non-meat foods and beverages with the cooked chicken. Control urine was collected before eating the chicken and samples were collected for 24 h after in increments of 6 h.

Urine samples were prepared according to Kulp et al. [46]. Briefly, an internal standard of deuterium labeled *N*-OH-PhIP-N^2-glucuronide was added to 5 ml samples of urine. The urine was then applied to a pre-conditioned macroporous polymeric column. Metabolites were eluted with methanol and the methanol fraction evaporated to dryness under nitrogen. The metabolites were re-dissolved in 0.01 M HCl and high molecular weight contaminants were removed by filtering the solution through a centrifugal filter at 3000 × *g* overnight. The filtrate was applied to a pre-conditioned benzenesulfonic acid column and the column washed with a mixture of methanol and 0.01 M HCl. The metabolites were eluted onto a coupled C18 column with 0.05 M ammonium acetate, pH 8. The C18 column was washed with 5% (v/v) methanol/H_2O and eluted from the C18 column with 50% (v/v) methanol/H_2O. The metabolites were dried under nitrogen and 1 ml urine equivalents were injected into the LC/MS/MS in a volume of 20 μl.

Metabolites were detected with an ion trap MS (model LCQ, Finnigan, San Jose, CA) in the MS/MS positive ion mode using an electrospray interface as published [47]. Alternating scans were used to isolate $[M + H]^+$ ions at mass 417, 401, and 321 for natural PhIP metabolites, and 422, for the pentadeutero-labeled internal standard metabolite. Collision energy was 25%. Daughter ions were detected at appropriate masses: 241 (M + H-glucuronic acid) and 225 (M + H-glucuronic acid-OH) from 417 for the *N*-OH-N^2 and N^3-glucuronide, respectively, 225 (M + H-glucuronic acid) from 401 for the PhIP-N^2-glucuronide, 241 (M + H-SO_3) from 321 for PhIP-4′-sulfate, and 246 (M + H-glucuronic acid) and 230 (M + H-glucuronic acid-OH) from 422 for the internal standard, *N*-OH-(D_5-phenyl)PhIP-N^2-glucuronide.

In Fig. 3, the rate of the excretion of PhIP urinary metabolites is shown. These data illustrate that with the exception of volunteer 3, the volunteers excreted more metabolites during the first 6 h after the broccoli intervention compared to the baseline level determined 1 week prior. Individual differences seen at different times may be due to differences in diet, which was uncontrolled except for the cooked meat and broccoli ingestion. Broccoli contains isothiocyanates, which have been shown to induce both cytochrome P450 enzymes

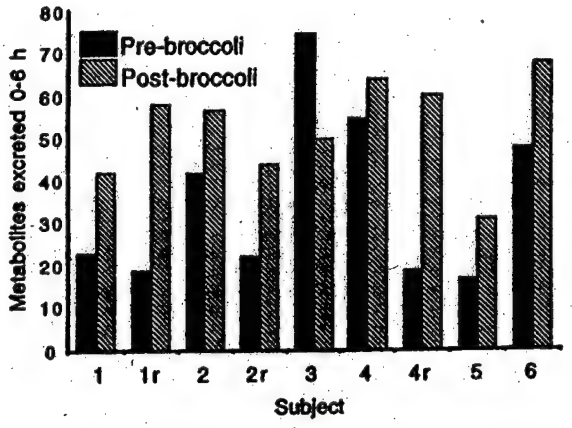

Fig. 3. Percent metabolites excreted in the first 6 h after eating chicken in six human volunteers either after abstaining from broccoli for 3 days (pre-broccoli) or after eating at least one cup of cooked broccoli for 3 days (post-broccoli). Three individuals were assayed twice; "r" signifies repeat. Five of six individuals excreted more of the metabolites after broccoli consumption, implying induction of metabolizing enzymes.

and glucuronyl transferases [48]. Our data suggest that broccoli may be affecting the rate of PhIP metabolism, because of the increase in the fraction of metabolites excreted in the 0–6 h time period.

There are many foods implicated in inducing or inhibiting carcinogen metabolizing enzymes. The PhIP urinary metabolite assay described above is designed to determine the influence of such foods on a dietary carcinogen at dietary doses in people. Because the metabolites are present in the urine at nanograms per milliters levels the assay poses several analytical difficulties. Extensive sample clean-up must be done to identify and quantify the metabolites above the background inherent in the urine sample and to prevent HPLC column degradation. The assay can still be improved in several ways. Heavy-isotope labeled metabolites are necessary for recovery determination of the *N*-OH-N^3-PhIP-glucuronide, PhIP-N^2-glucuronide, and PhIP-4′-sulfate. Additional PhIP metabolites are known to be present in human urine but have not yet been fully characterized. Although the unknown metabolites occur in smaller amounts than the four detected, quantifying these metabolites would provide a more complete picture of biological fate of the PhIP ingested in the chicken meal. Recently available mass spectrometers have about 10-fold more sensitivity than the current model, which might lead to improved peak signal, thereby reducing injection-to-injection variability.

5. A biomarker of heterocyclic amine exposure is still needed

To understand the effect of heterocyclic amine exposure on human health, we need to be able to assess actual exposures from meat prepared as it is commonly eaten in homes. Although measuring urine metabolites is one way of characterizing metabolism patterns, the metabolites excreted in the urine only represent exposures that may have occurred in the previous 24 h. The optimal biomarker of exposure would integrate heterocyclic amine exposures over time. Hair has been investigated as a marker of PhIP exposure over the previous 6 months [49].

Aflatoxin exposure assessment presents complexities similar to the heterocyclic amine exposure assessment. It sometimes occurs in only some foods, so the food contamination and amount eaten are both important for dose determination. A biomarker of exposure is available for aflatoxin, but a dietary questionnaire showed no positive correlation with measurement of blood serum levels of the AFB1-albumin adduct [50]. A biomarker would help judge if dietary questionnaires are useful for determining heterocylic amine intake. But a questionnaire and biomarker measurement are contemporary. Perhaps what is really needed is data regarding intake 20 years ago for individuals, or perhaps the heterocyclic amine intake during more sensitive adolescent years.

6. Conclusions

Intake variation of heterocyclic amines is suggested to be three orders of magnitude above the limit of detection from restaurant data for steaks [32] in the US. This seems to be a useful range in which to group human exposures and their cancer incidences.

The idea that the "the dose makes the poison" is important and may be relevant for dietary exposures to carcinogenic heterocyclic amines. It is possible that all heterocyclic amine doses are below the dose needed to show an effect. However, no evidence for threshold effects or non-linearity of DNA adducts exists for MeIQx in either rodents or humans [51].

The goal of understanding and reducing cancer is worthwhile. It requires understanding the tumor initiating mechanisms and controlling the relevant influences in epidemiology investigations. The heterocyclic amines are the perfect model compounds for both the basic and applied research, and results can be directly transferred to humans. The data are not currently available to fully characterize the relationship between heterocyclic amines and human cancer.

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Human Exposure to Heterocyclic Amine Food Mutagens/ Carcinogens: Relevance to Breast Cancer

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Heterocyclic amines produced from overcooked foods are extremely mutagenic in numerous in vitro and in vivo test systems. One of these mutagens, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), induces breast tumors in rats and has been implicated in dietary epidemiology studies as raising the risk of breast cancer in humans. Efforts in our laboratory and others have centered on defining the exposure to PhIP and other dietary mutagens derived from cooked food. We accomplish this by analyzing the foods with a series of solid-phase extractions and HPLC. We have developed an LC/MS/MS method to analyze the four major human PhIP metabolites (sulfates and glucuronides) following a single meal containing 27 μg of cooking-produced PhIP in 200 g of grilled meat. Although the intake of PhIP was similar for each of eight women, the total amount excreted in the urine and the metabolite profiles differed among the subjects. It appears that adsorption (digestion) from the meat matrix, other foods in the diet, and genetic differences in metabolism may contribute to the variation. The four major metabolites that can be routinely assayed in the urine are N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, 4′-PhIP-glucuronide, and N^2-OH-PhIP-N3-glucuronide. This work is suited to investigate individual exposure and risk, especially for breast cancer, from these potent dietary mutagens. Environ. Mol. Mutagen. 39:112–118, 2002. Published 2002 Wiley-Liss, Inc.†

Key words: dietary mutagen; heterocyclic aromatic amines; glucuronide; PhIP; tumorigenicity; chemoprevention

INTRODUCTION

The cooking, heat processing, and pyrolysis of protein-rich foods result in the formation of a group of structurally related heterocyclic aromatic amines that are potent mutagens in a number of assay systems. These same compounds produce tumors at multiple organ sites (including sites of important neoplasms in North Americans) in both male and female mice and rats [Shirai et al., 1997; Sugimura, 1997]. Furthermore, 100% of nonhuman primates given one of these heterocyclic amines (2-amino-3-methylimidazo[4,5-*f*]quinoline [IQ]) developed hepatocarcinomas after a very short latency period [Adamson et al., 1990, 1994]. Epidemiology data from a number of studies in the United States, New Zealand, South America, and Europe suggest a good correlation of meat consumption with cancer risk in humans. At a recent American Association for Cancer Research (AACR) meeting, there were four positive reports (three for breast cancer) relating high meat intake and genetic susceptibility with human cancer (8.2 relative risk for breast cancer when low Sult1A1 [Zheng et al., 2000], 3.5 odds ratio for breast cancer when rapid NAT2 [Visvanathan et al., 2000], and 1.9 odds ratio for breast cancer in the highest exposure group [Sinha et al., 2000]). It is now clear from a number of recent studies that these heterocyclic amines are present in the diet at higher levels than were originally anticipated [Knize et al., 1998]. The usual factor correlated with meat consumption and cancer occurrence is fat intake, but clearly, heterocyclic amine intake also correlates well and has a plausible genotoxic mechanism, leading directly to DNA binding, mutation, and cancer initiation.

Abbreviations: AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole (CAS no. 26148-68-5); 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (CAS no. 95896-78-9); 8-MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (CAS no. 77500-04-0); DMIP, 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine; IFP, 2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline (CAS no. 76180-96-6); IQx, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (CAS no. 108354-47-8); MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (CAS no. 77094-11-2); PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (CAS no. 105650-23-5); TMIP, 2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine; CHO, Chinese hamster ovary cells.

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Given these very compelling data, it is important to determine the extent to which these dietary mutagens/carcinogens contribute to human breast cancer incidence and to devise strategies to limit their impact. In this report we discuss the exposure with emphasis on heterocyclic amines in restaurant-cooked foods. We also discuss the risk of exposure to heterocyclic amines, metabolism with emphasis on glucuronyl transferases, urine metabolite biomarkers, and their possible use in evaluating risk for breast cancer from these carcinogens found in cooked meat.

HETEROCYCLIC AMINE ANALYSIS OF FOODS

Twenty years ago, the chemicals responsible for the observed mutagenic activity in cooked meat were unknown. Discoveries in the dietary heterocyclic amine field date back more than 24 years [Sugimura et al., 1977]. Dr. Sugimura and his group first showed that cooking of meat and fish produced potent bacterial mutagens [Sugimura et al., 1977]. Dr. Kasai, working with Drs. Sugimura and Nishimura, described the structure of the first mutagen isolated from cooked meat (IQ) [Kasai et al., 1981]. Shortly after this initial work, our group quantified the level of mutagenic activity in numerous food types in the Western diet. We later isolated and identified from cooked ground beef IQ, MeIQx, and, for the first time, DiMeIQx and PhIP [for review, see Felton et al., 1986; Felton and Knize, 1991; Felton, 1994]. We also determined that PhIP was present at approximately 10-fold higher mass amounts than that of the other heterocyclic amine mutagens [Felton et al., 1986]. Our scientists partnered with researchers from the Nestlé Ltd. Research Centre to develop analytical methods for the practical detection of heterocyclic amines in foods, to determine the foods and cooking conditions responsible for human exposures [Gross and Grüter, 1992; Knize et al., 1992].

A few years later, liver tumors were observed in cynomolgus monkeys fed IQ [Adamson et al., 1990]. With the discovery of mutagenic responses of these heterocyclic amines in multiple genotoxic assay systems, and carcinogenicity responses in both sexes and multiple organs of rats [Sugimura et al., 1988], mice [Ohgaki et al., 1987; Esumi et al., 1989], and primates [Adamson et al., 1990], it became clear that these compounds had a potentially important impact on human health and, particularly, on cancer risk [Sugimura, 1997]. In one of the early human epidemiological studies, Schiffman and Felton [1990] described an increased relative risk for colon cancer for individuals consuming fried meats.

HUMAN RISK TO HETEROCYCLIC AMINES

Data have been reported on the levels of the heterocyclic amines in the diet [Fennema and Hall, 1990; Layton et al., 1995]. Several early studies on the epidemiology of these compounds [Gerhardsson de Verdier et al., 1991; Steineck et al., 1993; Goldbohm et al., 1994] showed a relationship between meat consumption and human cancer (see above for more recent epidemiology studies related specifically to breast cancer). Human risk, based on linear extrapolation of TD_{50} calculations from mouse, rat, or primate tumor data, and on mean estimated mutagen exposures for the U.S. population, suggests potential risks of 10^{-5} to 10^{-3} [Gaylor and Kadlubar, 1991; Layton et al., 1995]. Although these risk calculations contain many generalizations and assumptions, nevertheless they indicate that human risk from dietary ingestion of these heterocyclic amines may be significant. These risk estimates need to be supported or refuted using much more rigorous data and linked to specific human subpopulations that may be more susceptible or "at risk" than is the average population.

MUTAGENS IN THE DIET

Analysis of *Salmonella* mutagens in major sources of cooked protein in the American diet (based on USDA and USDHEW surveys) showed significant mutagen content in beef, eggs, pork, ham, and bacon, and lesser amounts in chicken and fish (fried or broiled) [Bjeldanes et al., 1982a]. Tofu, beans, cheese, and some fish, when cooked under similar conditions, produced low or negligible mutagenic activity [Bjeldanes et al., 1982b]. Mutagen isolation was improved by aqueous extraction at pH 2 followed by absorption/elution of mutagens on XAD-2 resin [Bjeldanes et al., 1982a]. Chromatographic purification of mutagens from 100-kg batches of fried beef was combined with high-resolution mass spectrometry and NMR techniques to show the presence of at least 10 separable mutagens. The largest amount of mutagenicity was provided by MeIQx (~35% of total activity), which is present at about 1 μg/kg original fresh weight of beef. Additional major mutagens were 4,8-DiMeIQx (0.5 μg/kg) and PhIP (15 μg/kg). Several other mutagens were present, including IQ (0.02 μg/kg), MeIQ (at <0.01 μg/kg), and TMIP (0.5 μg/kg) [Felton et al., 1984]. More recently, analytical methods were further improved with the development of GC/MS techniques and solid-phase extraction with HPLC analysis [Gross and Grüter, 1992; Knize et al., 1992]. This work has led to the finding that heterocyclic amine content in foods is significantly higher than was originally anticipated.

Mutagen production in beef, chicken, and pork has been examined at different temperatures. Even though total mutagenic activity increases dramatically with increasing temperature, chromatographic analysis shows that the relative amounts of the mutagenic peaks are similar [Knize et al., 1985]. Mutagen profiles from chicken breast meat (ground and then fried) is similar to, but not identical with, the beef mutagen profile [Knize et al., 1988]. Our early analysis (a collaboration with the group at Wageningen University, The Netherlands) of a complete human diet, with foods and amounts taken from a dietary survey and cooked under

TABLE I. Heterocyclic Amines in Restaurant Foods[a]

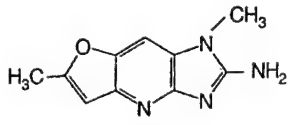
Sample	Restaurant—doneness	IFP	MeIQx	PhIP	DMIP	TMIP	DiMeIQx
Top sirloin	A—well-done	nd	1.2[b]	1.8	nd	nd	nd
New York steak	A—well-done	nd	0.12	0.86	nd	nd	nd
Pork chop	A—unspecified	nd	0.4	2.4	nd	nd	nd
Beef (French-dip sandwich)	A—unspecified	nd	nd	nd	nd	nd	nd
New York steak	B—well-done	7.0	1.3	7.7	7.2	1.5	0.77
Tenderloin steak #1	C—well-done	7.6	1.9	16	nd	nd	nd
Tenderloin steak #2	C—well-done	21	0.67	49	nd	nd	nd
Top sirloin steak	D—well-done	3.3	2.0	7.8	nd	nd	nd
London broil steak	C—well-done	46	3.0	182	3.4	nd	nd
Prime rib	C—well-done	nd	nd	nd	nd	nd	nd
Beef (fajitas)	D—unspecified	1.4	0.93	1.7	0.59	nd	0.06
Au jus gravy	A—unspecified	nd	nd	nd	nd	nd	nd

[a]ng heterocyclic amine/g cooked meat; nd, not detected.
[b]Average of duplicate analyses of a single sample.

"household" conditions, also shows chromatographic types and amounts of mutagens similar to those of fried beef [Alink et al., 1988]. More recent studies show that the amounts of these compounds formed increase exponentially with temperature, and the ultimate levels attained are dependent on cooking method, cooking time, cooking temperature, and protein source [Knize et al., 1994]. In fact, the levels in some foods, such as chicken, can reach hundreds of parts per billion [Sinha et al., 1995]. In general, these compounds are formed at surface temperatures in excess of 150°C and are found in all well-done broiled, grilled, or fried muscle meat products, including fish, beef, pork, and chicken. These heterocyclic amines have also been reported in cigarette smoke [Manabe et al., 1991] and wine and beer [Manabe et al., 1993], although these findings have not yet been confirmed in other laboratories. These results clearly indicate that cooked meats are the major source of heterocyclic amines in the human diet.

Most recently, we have analyzed restaurant-cooked foods to see how the levels of heterocyclic amines compare to those found from laboratory and home cooking. In Table I, we show a large range in heterocyclic amine content from different meats. In one restaurant, the level of PhIP was almost 200 ppb in London broil beef ordered well-done. In most cases, the levels were at least 10-fold below this highest level. Chicken, especially that grilled from a Mexican restaurant, was significantly high for a number of the heterocyclic amines. This study shows that exact concentrations of the heterocyclic amines in these cooked foods will be difficult to determine based only on questionnaires of doneness preference. However, it is clear from this study that significant amounts of heterocyclic amines can be consumed from eating commercially cooked well-done meats.

The identification of new mutagens from cooked meats has been difficult but successful. Several new mutagens have been identified, with structures consisting of two fused rings and either two or three methyl groups (DMIP and TMIP). Recently, a new mutagen with an imidazo-furo-pyridine structure has been found in a variety of meats (Table I) and its structure



IFP
(2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine)

Fig. 1. Structure of 2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine (IFP).

has been characterized as 2-amino-(1,6-dimethylfuro[3,2-*e*])imidazo[4,5-*b*]pyridine (see Fig. 1 for the structure). Its role in breast cancer and other human tumor sites is unknown at this time, but its potent mutagenic activity and its structural similarity to PhIP make investigation of its biological effects a priority.

HETEROCYCLIC AMINE MUTAGEN METABOLISM

The metabolism of PhIP and 4,8-DiMeIQx, two of the most mass-abundant heterocyclic amines, differing greatly in their mutagenic response in cultured CHO cell and Salmonella mutagenic responses, were investigated in both in vivo and in vitro rodent experiments. PhIP is metabolized to two major metabolites by mouse liver microsomes, one of which is a direct-acting mutagen (N-OH-PhIP) to *Salmonella* and CHO cells. The other metabolite is hydroxylated at the 4′ position of the phenyl ring and appears to be a detoxification product [Turteltaub et al., 1988]. Thus, it is important to understand factors that favor formation of one or the other of these metabolites because the ratio will affect the level of reactive intermediates available for DNA binding (adduct formation) and mutation.

In Aroclor 1254–induced C57BL/6 mice, PhIP is excreted almost completely in 24 hr, with some differences in its uptake kinetics between oral and intraperitoneal administration. The urine shows at least six metabolites, with less than 10% of the dose excreted as the unaltered parent compound [Turteltaub et

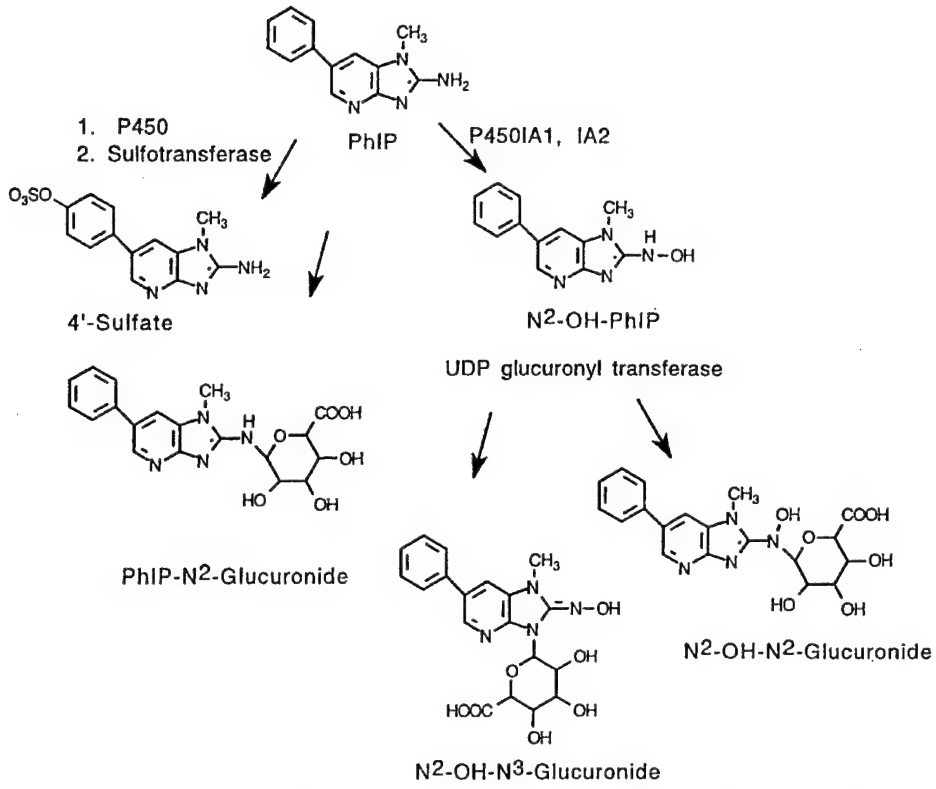


Fig. 2. Structures of PhIP metabolites and pathways of their formation. These metabolites are identified in human urine after consumption of a single meal of well-done chicken. The major metabolites are N^2-OH-PhIP-N^2-glucuronide (conjugation of an active metabolite) and PhIP-N^2-glucuronide (a conjugated detoxification product).

al., 1989]. 4,8-DiMeIQx is metabolized to eight metabolites by microsomes in vitro. Two of these have been identified as nitro-4,8-DiMeIQx, which is possibly a degradation product of N-hydroxy-4,8-DiMeIQx and 8-hydroxymethyl-4,8-DiMeIQx [Turteltaub et al., 1988].

In rats, seven major 4,8-DiMeIQx metabolites were detected in the urine and feces. Germ-free rats, having no intestinal microflora, produced the same group of metabolites in both the urine and feces as did the normal rats [Knize et al., 1989]. This suggests that microbial metabolism is not a significant factor in the metabolism of this mutagen.

It seems clear from in vitro studies that acetylation is required for the formation of active electrophiles of IQ and MeIQx, but not of PhIP. Mutagenicity of N-hydroxy-PhIP depends somewhat on bacterial sulfotransferase activity but not on acetylation. IQ and MeIQx, but not PhIP, were significantly less mutagenic in *Salmonella* strains that had a deficiency in *N*-acetyltransferase activity [Holme et al., 1989; Buonarati et al., 1990]. In collaboration with Dr. Josephy (Guelph University, Canada), we also showed that strains overexpressing *N*-acetyltransferase were more responsive to IQ but not to PhIP (unpublished data). It appears that N-OH intermediates of these amines have different requirements for conjugation and these differences may explain variable responses in CHO cells and tissue-specific carcinogenicity differences. Further, human tissue cytosols catalyze both N:O-acetylation and N:O-sulfation, but the in vivo rates of metabolism have yet to be determined. More recently, strains overexpressing sulfotransferase showed the biggest increase in mutation and cytotoxicity with PhIP [Wu et al., 2000], but nowhere near the large response seen with IQ when acetyltransferase is overexpressed [Wu et al., 1997]. These differences need to be explored to understand individual differences in biological response, especially in the tumor targets for these heterocyclic amines, such as breast tissue.

METABOLISM IN HUMANS

Following cytochrome P4501A2 activation of the parent amine to the corresponding 2-hydroxyamino intermediate, a number of conjugating reactions can take place [Boobis et al., 1994; Edwards et al., 1994]. For PhIP, the N-hydroxy intermediate can be esterified by sulfotransferase and/or

acetyltransferase to generate the highly electrophilic O-sulfonyl and O-acetyl esters [Buonarati et al., 1990; Ozawa et al., 1994]. Most interestingly, human metabolism of PhIP is dominated by glucuronidation (see Fig. 2) [Malfatti et al., 1999]. In addition, understanding of glucuronidation by a family of enzymes called the UDP-glucuronosyltransferases (UGTs) is needed. These enzymes exist as a number of different isoforms [King et al., 1996; Mackenzie et al., 1997; Strassburg et al., 1998], but the UGT1A subfamily contributes to the biotransformation of amines and PhIP, respectively [Green and Tephly, 1998; Nowell et al., 1999]. Microsomes containing the UGT1A1 isozyme have the highest capacity to convert *N*-hydroxy-PhIP to *N*-hydroxy-PhIP-N^2-glucuronide, the most abundant metabolite in human urine formed from PhIP [Malfatti et al., 2001]. In contrast, UGT1A9 produced *N*-hydroxy-PhIP-*N*3-glucuronide at the highest rate. Thus, the distribution and prevalence of these isozymes in the body may determine the rate and type of detoxification of PhIP and, ultimately, the target tissue for mutations and cancer.

Both the N^2 and the *N*3 positions on PhIP are glucuronidated directly (most likely these are nonreactive intermediates) or the glucuronidation occurs on the N-hydroxy intermediates. This can be envisioned as a direct detoxification pathway (see Fig. 2) [Styczynski et al., 1993; Kaderlik et al., 1994]. These glucuronides and the 4′ sulfation product on the phenyl ring of PhIP can be accurately measured in human urine using LC/MS/MS, after a single meal of cooked well-done meat [Kulp et al., 2000]. The ratios of these metabolites can be measured to understand individual differences in metabolism, and also can be used to determine whether chemopreventative agents can alter the metabolism of these mutagens (see below).

CHEMOPREVENTION IN HUMANS

With the ability to measure PhIP metabolites in humans, we can do intervention studies to determine whether chemopreventative agents, such as isothiocyanates in broccoli, can alter the metabolism (and possibly the risks) from exposure to these agents. Six volunteers were fed a single meal of well-done chicken after abstaining from broccoli or related cruciferous vegetables for 3 days. Metabolites were determined in urine collected in 6-hr increments. N^2-OH-PHIP-N^2-glucuronide was the primary PhIP conjugate detected in human urine after the chicken meal. The N^2-hydroxy-PhIP-*N*3-glucuronide, the PhIP-N^2-glucuronide, and the PhIP-4′-sulfate were the other major metabolites. After eating cooked broccoli for 3 days, the experiment with well-done chicken was repeated. As shown in Figure 3, metabolism of PhIP to the conjugates detected in the first 6 hr was increased statistically in all but one individual after the broccoli consumption. This suggests that components in the broccoli increased the rate of PhIP metabolism.

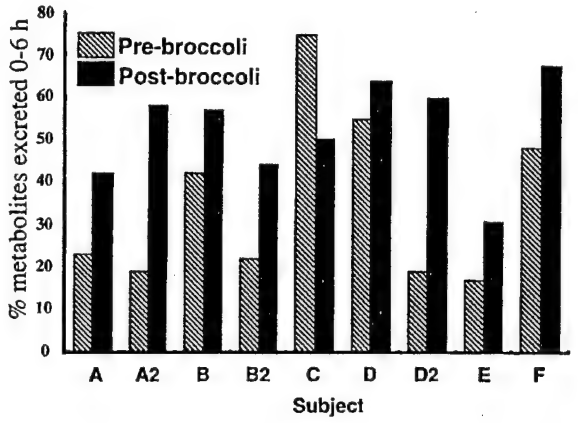


Fig. 3. Percentage of the measurable PhIP metabolites excreted in the 0–6 hr time period in six individuals after abstaining from broccoli-related cruciferous vegetables (pre-broccoli) or after consuming broccoli daily for 3 days (post-broccoli). Volunteers were given a single meal containing well-done chicken breast, cut into 2.5-cm pieces fried 25–35 min at an average pan temperature of 186°C. Total PhIP doses were 10–20 μg. Three individuals were assayed twice (2 represents repeat on the same individual 3 months later). The increase in excreted metabolites may be attributable to induction of phase II metabolizing enzymes by compounds such as isothiocyanates in the broccoli.

CONCLUSIONS

The investigation of the heterocyclic amines and their human intake is important for breast cancer research, for several reasons. From epidemiology studies, breast cancer is relatively high among women eating a Western diet, which is consistent with consumption of cooked meat (beef, chicken, pork, fish, and lamb) foods. One known heterocyclic amine, PhIP, consistently causes mammary tumors in rats, although IQ and Trp-P-2 induce mammary tumors as well in Sprague–Dawley and F344 rats, respectively. International studies show that PhIP is present in well-done meats, whether consumed in homes or in restaurants.

Under continuing investigation are the differences in heterocyclic amine metabolism, comparing and extrapolating rat tumorigenicity to humans. Extrapolation from high-dose animal experiments to the low doses found in human studies is difficult, but still there are compelling data to suggest heterocyclic amines may be good model compounds for investigation of breast cancer initiation in humans. Because we can measure carcinogenic metabolites in people, we can go forward with chemoprevention studies in humans, especially those relevant for breast cancer.

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Liquid chromatography–tandem mass spectrometry method of urine analysis for determining human variation in carcinogen metabolism

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Abstract

We developed a solid-phase extraction LC–MS–MS method for the analysis of the four major metabolites of PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) in human urine after a meal of well-done chicken. Ten volunteers each ate either 150 or 200 g of well-done chicken breast containing 9–21 μg of PhIP. Among the individual volunteers there is 8-fold variation in the total amount of metabolites and 20-fold variation in the relative amounts of individual metabolites, showing individual differences in carcinogen metabolism. PhIP metabolites were also detected in urine from a subject consuming chicken in a restaurant meal, demonstrating the method's sensitivity after real-life exposures. Published by Elsevier Science B.V.

Keywords: Amines, heterocyclic aromatic; Aminomethylphenylimidazo[4,5-*b*]pyridine; Pyridines; Glucuronides

1. Introduction

PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine) is a potent mutagen and rodent carcinogen formed in meats from natural precursors during the cooking process. PhIP is found at the highest levels in grilled or fried meats and is frequently the most mass abundant heterocyclic amine produced during the cooking of beef, pork, and chicken [1–5], and in meats cooked by professional chefs and purchased in restaurants [6,7]. The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day, depending on individual dietary and cooking preferences [8]. Because humans are routinely exposed to varying amounts of these food-derived compounds there is a concern that they may play a role in human carcinogenesis.

PhIP must first be metabolized via Phase I and Phase II enzymes to exert its mutagenic and carcinogenic effect. During Phase I metabolism PhIP is oxidized to a hydroxylated intermediate, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-hydroxy-PhIP). *N*-hydroxy-PhIP is then converted to a more biologically reactive form via Phase II metabolizing enzymes, primarily the acetyltransferases or sulfotransferases. PhIP can also be hydroxylated at the 4′ position, forming 2-amino-1-methyl-6-(4′-hydroxy) phenylimidazo[4,5-*b*]pyridine (4′-hydroxy-PhIP). This hydroxylation does not produce an active intermediate. 4′-Hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted. Detoxification primarily involves glucuronidation. *N*-Hydroxy-PhIP can form stable glucuronide conjugates at either the N^2 or *N*3 positions. In addition, the parent compound can be directly glucuronidated at the N^2 and *N*3

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positions. These glucuronides are not reactive and are excreted in the urine.

There is conclusive evidence that PhIP, a genotoxic carcinogen, is involved in tumorigenesis in animals. In rats and mice, dose-dependent tumor formation has been consistently demonstrated after PhIP administration, and the most common tumor sites in the rat appear to be colon, prostate, and breast [9–14].

Less is known about the role of PhIP in human carcinogenesis. Until recently, studies of human PhIP metabolism have been limited to hepatic microsomes or cells in culture. Pioneering studies in in vivo human metabolism demonstrated the presence of PhIP and PhIP conjugates in human urine, but in these studies the urine was first treated with acid to hydrolyze the Phase II conjugates to the parent amine. These investigations proved that PhIP is bioavailable in humans, but did not give information about specific metabolic pathways [15,16]. Specific results about the identity of human PhIP metabolites were obtained in studies that investigated human PhIP metabolism following administration of ^{14}C-labeled PhIP to patients undergoing cancer surgery. We recently described human PhIP metabolism in cancer patients receiving a single dose of radiolabeled PhIP in a capsule. These studies identified four major human PhIP metabolites: N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, PhIP-4′-sulfate, and N^2-OH-PhIP-N3-glucuronide [17].

In the present study we describe our development of a solid-phase extraction LC–MS–MS method for quantifying the four most abundant PhIP metabolites in human urine, following a meal of well-cooked chicken. We applied this method to characterize PhIP metabolism in 10 healthy individuals receiving a known dose of naturally produced PhIP. We have also extended this method to monitor metabolite excretion in a subject consuming chicken as part of a restaurant meal, demonstrating that our method is sensitive enough to detect PhIP metabolites after common real-life exposures.

2. Material and methods

2.1. Synthesis of N^2-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide internal standard

The biological synthesis of deuterium labeled N-OH-PhIP-N^2-glucuronide was carried out in two steps as described previously [18]. Briefly, pentadeutero PhIP was reacted with baculovirus infected insect cell microsomes expressing human cytochrome P4501A2 (Gentest, Woburn, MA, USA) to produce the N-OH-[$^2H_5$-phenyl]PhIP intermediate. The reaction products were concentrated under N_2 and then isolated by HPLC using a Waters Alliance HPLC system equipped with a 5 μm, 220×4.6 mm TSK-Gel ODS-80 TM column (TosoHaas, Montgomeryville, PA, USA). Metabolites were detected using a Waters 990 photodiode array detector. The N-OH-[$^2H_5$-phenyl]PhIP was eluted at 1.0 ml/min using a gradient starting at 30% aqueous methanol, 0.1% triethylamine, pH 6, to 55% aqueous methanol, 0.1% triethylamine, pH 6, at 8 min. The methanol concentration was maintained at 55% from 8 to 20 min. After evaporation of the mobile phase, the yield of N-OH-[$^2H_5$-phenyl]PhIP from [$^2H_5$-phenyl]PhIP was approximately 40%.

Purified N-OH-[$^2H_5$-phenyl]PhIP was reacted with microsomes derived from the AHH-1 TK+/-human lymphoblastoid cell line which expresses human UDP-glucuronosyltransferase 1A1 (Gentest). The N-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide was isolated and purified by HPLC using the conditions described above to give a 15% yield from N-OH-[$^2H_5$-phenyl]PhIP.

2.2. Study design

The study protocol was reviewed and approved by the Institutional Review Board for Human Research at Lawrence Livermore National Laboratory. Informed consent was obtained from each subject prior to beginning the study. The individuals participating were recruited from the local workforce, were males and females aged 22–45 years, in good health, nonsmokers, and of normal weight.

2.3. Meat preparation and controlled dietary period

Boneless, skinless chicken breasts were cut into approximately 2.5 cm pieces and fried for 25 to 35 min in a non-stick coated pan sprayed with a vegetable-based cooking spray. Pan temperature averaged 186°C for the cooking period. At the end of the cooking time the chicken was white with some

browning. PhIP analysis was performed according to previously published methods [19].

Subjects were asked to abstain from meat consumption for 24 h prior to eating the well-done chicken breast. There were no other dietary restrictions. The first two study subjects were provided with 200 g chicken containing 105 ng/g PhIP. The total PhIP dose was 21 μg. Subjects three to eight were given 200 g of chicken containing 94 ng/g PhIP, for a total dose of 18.8 μg. The remaining two subjects were given 150 g of chicken containing 62 ng/g PhIP, for a total dose of 9.2 μg. All subjects were provided with other non-meat foods and beverages with the cooked chicken.

Control urine was collected before eating the chicken and for 24 h after in 6 h increments. Samples were refrigerated until analysis. Repeated analysis of these samples over prolonged periods of time (greater than 1 year) have shown no noticeable change in metabolite levels.

2.4. PhIP metabolite analysis after a restaurant meal

To test the sensitivity of detection of this method, one subject ordered and consumed chicken that was prepared as "chicken mango" at a local restaurant. The subject ate approximately 80 g of grilled chicken containing 33 ng/g of PhIP (a portion of the entrée was reserved and later analyzed using previously published methods [19]). Urine was collected for approximately 4 h, 4–8 h after eating the meal.

2.5. Extraction of PhIP metabolites

Urine samples (5 ml) were spiked with internal standard (4.2 ng, in 5 μl water) and applied to a pre-conditioned 60 mg Oasis SPE macroporous polymeric column (Waters, Milford, MA, USA). Metabolites were eluted with 5 ml methanol. The elution aliquot was evaporated to dryness under nitrogen and the metabolites were re-dissolved in 2.5 ml 0.01 *M* HCl. Proteins and high-molecular-mass contaminants were removed by filtering the solution through a Centricon YM-3 centrifugal filter (Millipore, Bedford, MA, USA). The samples were centrifuged in the filter at 3000 *g*, overnight. The filtrate was applied to a pre-conditioned benzenesulfonic acid column (SCX, 500 mg, Varian, Harbor City, CA, USA) and the column washed with 6 ml of 10% (v/v) methanol in 0.01 *M* aqueous HCl. The metabolites were eluted onto a coupled C_{18} column (Baker-bond spe, 1000 mg, J.T. Baker, Phillipsburg, NJ, USA) with 0.05 *M* ammonium acetate, pH 8. The C_{18} column was washed with 3 ml of methanol–water (5:95, v/v) and eluted from the C_{18} column with methanol–water (50:50, v/v). The metabolites were dried under nitrogen and 1 ml urine equivalent was injected into the LC–MS–MS in a volume of 20 μl.

Chromatography was done on a Microtech Ultra-Plus HPLC system (Sunnyvale, CA, USA) equipped with a YMC ODS-A column (250×3.0 mm). Metabolites were eluted at a flow-rate of 200 μl/min using a mobile phase of A (water–methanol–acetic acid, 97:2:1) and 5% B (methanol–water–acetic acid, 95:4:1) for 1 min, to 25% B at 5 min, and a linear gradient to 100% B at 30 min and held for 5 min.

Analytes were detected with an ion trap mass spectrometer (model LCQ, Finnigan, San Jose, CA, USA) in the MS–MS positive ion mode using an electrospray interface. The capillary temperature was 240°C and the spray voltage was 4.5 kV. The sheath gas was set at 70 units and no auxiliary gases were used. The ion trap injection time was 1000 ms and a setting of one microscan was used.

Alternating scans were used to isolate $[M+H]^+$ ions at mass 417, 401, and 321 for natural PhIP metabolites, and 422, for the pentadeutero-labeled internal standard metabolite. Collision energy was 25%. Daughter ions were detected at appropriate masses: 241 $[M+H\text{-glucuronic acid}]^+$ and 225 $[M+H\text{-glucuronic acid-OH}]^+$ from 417 for the *N*-hydroxy-N^2 and *N*3 glucuronide, respectively, 225 $[M+H\text{-glucuronic acid}]^+$ from 401 for the PhIP N^2 glucuronide, 241 $[M+H\text{-}SO_3]^+$ from 321 for PhIP-4′-sulfate, and 246 $[M+H\text{-glucuronic acid}]^+$ and 230 $[M+H\text{-glucuronic acid-OH}]^+$ from 422 for the internal standard, *N*-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide. An external standard of naringenin was used in later samples, its $[M+H]^+$ ion isolated at mass 273 with protonated fragments detected at mass 147, 153, and 185.

2.6. Recovery studies and precision of the assay

The overall recovery of the metabolites was determined by spiking each urine sample with known amounts of *N*-OH-[$^2H_5$]PhIP-N^2-glucuronide. Final metabolite amounts were adjusted based on the recovery of the internal standard. The effect of the urine matrix on the recovery of the metabolites was determined by spiking increasing amounts of the internal standard in 5 ml of water and comparing these recoveries to the recovery of the internal standard in 5 ml urine.

Ion suppression in the mass spectrometer by co-eluting interferences was investigated by spiking human urine extracts with mouse urine containing high levels of metabolites. In our method, the *N*-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide is used as a surrogate standard for all of the metabolites because of the structural similarity of the metabolites and our belief that it is representative of the other metabolites, within the precision of other aspects of our assay. An external standard of naringenin added to later samples shows that ion suppression is consistent and suppresses the signal by 65% compared to the external standard injected alone.

Replicate analyses of several different urine samples were made during the course of the study to determine the precision of the assay. The coefficient of variation was approximately 28% for urine extractions and LC–MS–MS, with much of the variation occurring in the LC–MS–MS instrument. Consequently, samples were injected three times and the results averaged.

3. Results and discussion

3.1. Method development and urine analysis

The goal of this work was to develop a method that reliably quantifies PhIP metabolites and could be applied to large numbers of urine samples. The initial step of the method utilizes non-specific adsorption to remove all the metabolites from the water and salts in the urine. Other materials were tried in preliminary work, such as C_4, C_8, and C_{18} solid-phase extraction materials and styrenedivinylbenzene medium packed into columns, but none recovered all four metabolites as well as the polymeric material in the Oasis columns.

Our initial attempts at sample clean-up resulted in samples that did not chromatograph well. Poor HPLC column lifetime, peak broadening, and increasing retention time for two of the metabolites, N^2-OH-PhIP-N^2-glucuronide and PhIP-N^2-glucuronide were the symptoms of this problem. Suspecting that urinary proteins and larger molecule contaminants were the cause of some of these symptoms, they were removed by centrifuging the extracts through a filter with a molecular mass cut-off of 3×10^6. Protein determinations of the urine samples before and after filtering demonstrated that 60–80% of the color-reacting material could be removed from the sample during the filtering step (data not shown). This improved HPLC column lifetimes somewhat. After the centrifugation step, further purifications exploited the protonation of the heterocyclic nitrogen atoms that are common to the all the metabolites. This ion-exchange adsorption step was designed to remove uncharged interferences. Finally, the urine extract was concentrated and washed on reversed-phase silica.

To monitor the recovery of the metabolites through the method, a deuterium-labeled internal standard is added to the urine before extraction. Typical recoveries range from 37 to 40%. Final metabolite levels for each sample were adjusted based upon the recovery of the internal standard in that sample. Because of the small peak sizes in the assay, there is variation inherent in the mass spectrometry detection. To account for this variation, each urine extract was injected three times and the peak areas averaged. Variation within samples ranged from 20 to 30%.

Because of the complexity of the urine extracts and the low amounts of metabolite present, metabolites could not be seen by UV or fluorescence detection. Mass spectrometry must be employed.

Urine samples from rodents receiving high doses of PhIP were used to optimize the HPLC separation and the fragmentation of the metabolites. Metabolites in rodent urine were used to determine the linear range of the instrument. The LC–MS–MS peak areas were linear over the range of peaks seen in this study, which is approximately 20-fold higher than the limit of detection. Internal calibration curves

were calculated for each metabolite based upon rodent urine spiked into a human urine matrix. R^2 values were: N^2-OH-PhIP-N^2-glucuronide, 0.9703, PhIP-N^2-glucuronide, 0.978, PhIP-4′-sulfate, 0.999, and N^2-OH-PhIP-N^3-glucuronide, 0.9954.

Further, because of the co-elution of hundreds of compounds into the mass spectrometer, no signal can be seen above the background with single ion monitoring MS for the parent masses (Fig. 1A). MS–MS detection is necessary for these analyses. Fig. 1B shows a human urine sample analyzed by LC–MS–MS, showing peaks for the fragments of four metabolites after the isolation of the parent masses.

Volunteers are asked to refrain from eating meat for 24 h before eating the cooked chicken, and a control urine sample is collected at the end of the meat-free period. A chromatogram that represents a typical sample of control urine is provided in Fig. 2A. No metabolite peaks are seen at the retention times of PhIP metabolites. Fig. 2B represents urine from the same individual, collected during the first 6 h after consuming the chicken. Peaks are clearly seen for each of the four PhIP metabolites.

Fig. 3 shows the percentage that each individual metabolite represents of the total of all metabolites excreted over 24 h for 10 individuals. The N^2-OH-PhIP-N^2-glucuronide was the major metabolite in all cases. PhIP-N^2-glucuronide is the second most abundant, but the ratio of these two metabolites varies from almost equal amounts for subject 2 to 9-fold more N^2-OH-PhIP-N^2-glucuronide in subject 6. With the exception of subject number 10, N^2-OH-PhIP-N^2-glucuronide and PhIP-N^2-glucuronide together account for 90% or greater of the total metabolite excreted. Subject 10 excreted a much higher proportional amount of N^2-OH-PhIP-$N3$-glucuronide (22%) in contrast to the other individuals, in whom N^2-OH-PhIP-$N3$ glucuronide accounted for 7% or less of the total metabolite excreted. The time of excretion of metabolites also varies (data not shown), with some individuals excreting most of the metabolites in the 0–6 h time period and some later, in the 6–12 h time period. Little or no metabolite is detected in the 18–24 h time period.

To extend our method to real-life exposures, we collected urine from an individual who had consumed chicken as part of a restaurant meal. Fig. 4 shows the LC–MS–MS chromatogram of a urine extract collected 4–8 h after consuming the meal. Peaks for all four metabolites and the deuterium-labeled internal standard can be detected.

Our method provides an opportunity to study a genotoxic dietary carcinogen at realistic levels in humans. PhIP is of special interest because it causes tumors in animals that are among the most common cancer sites in humans: the breast, colon, and prostate gland. In addition, exposure to PhIP need not be ubiquitous, but can be determined and modified through intervention, making PhIP-induced tumor formation preventable.

Several different types of studies can be supported by this analysis method. Relative amounts of PhIP metabolites can be used to determine individual metabolic phenotype. The effect of diet on carcinogen metabolism can be determined by controlled feeding studies that analyze the changes in the relative amounts and time of excretion of metabolites. Urine metabolites can also be quantified for individuals on a normal diet, to monitor for exposure levels.

The enzymes known to be involved in the metabolism of PhIP are found at varying levels and activities within the human population [20]. The expression of specific activating enzymes has a great affect on the biological reactivity of PhIP. We believe that the N^2-OH-PhIP-N^2-glucuronide and N^2-OH-PhIP-$N3$-glucuronide metabolites represent the metabolic products of activation pathways, whereas the PhIP-N^2-glucuronide and 4′-PhIP-sulfate represent detoxification pathways. The variation that we detect in these metabolites suggests that the levels of both activation and detoxification enzymes varies among individual volunteers and may be a way to quantify individual phenotype or genotype. Using our method to generate a metabolic profile could provide an indication of potential susceptibility to DNA damage, mutation, and cancer.

On possible mechanism for the protective effects of fruits and vegetables seen in human cancer studies is the influence of natural compounds on both primary and secondary metabolism. This suggests that the metabolism of carcinogens, including PhIP, can be modified by the addition of protective foods to the diet. Our method provides an invaluable tool for monitoring the effect dietary interactions on PhIP

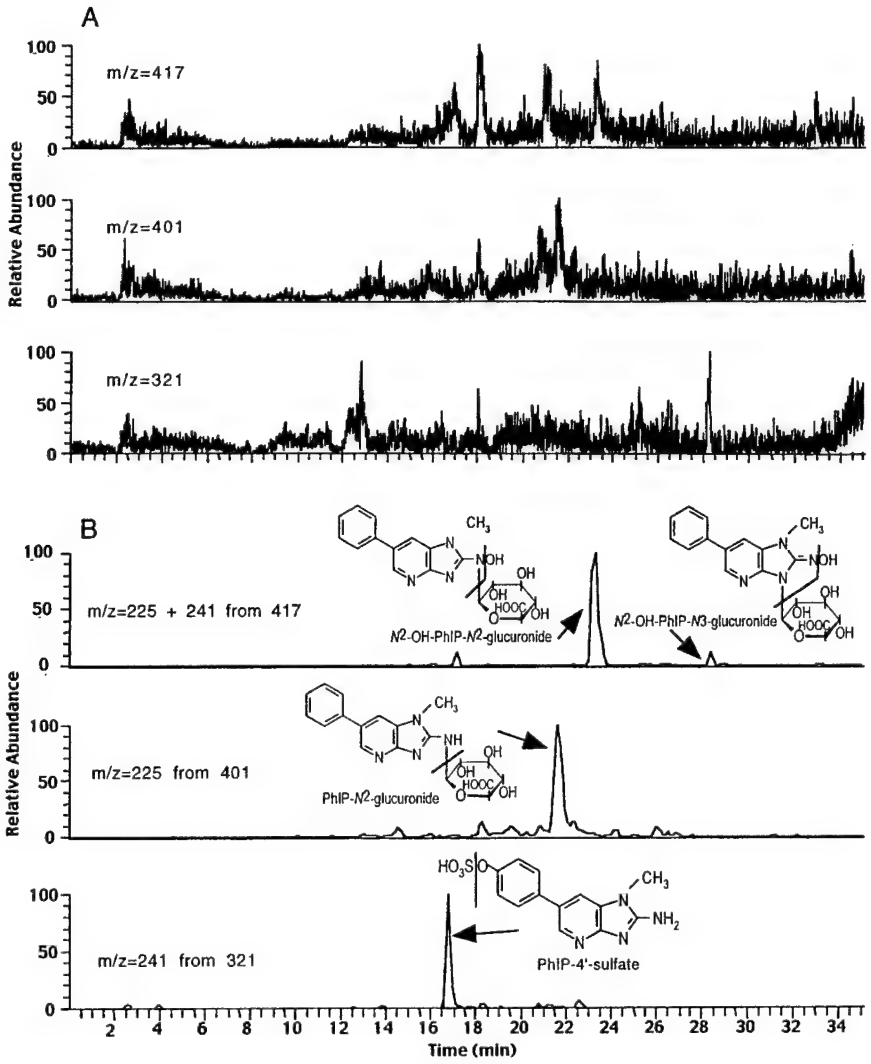

Fig. 1. Reversed-phase HPLC mass chromatograms of urine extract. (A) shows full scan plot of the *m/z* 417, 401 and 321 corresponding to PhIP metabolites. (B) MS–MS chromatograms of the human urine sample with masses isolated as indicated. Peaks are clearly seen for four metabolites indicated by arrows. Chemical structures and a line indicating the site of fragmentation for each structure are shown.

metabolism. These effects on metabolism can be quantified in humans at normal dietary levels using our method.

Determining the dietary dose of PhIP is important for epidemiology studies and risk determination. Typically, exposure estimations are made through dietary questionnaires. However, the formation of PhIP is variable, and the amount in foods depends on the cooking methods. Dietary surveys have several flaws, including bias, inconsistent reporting, and most importantly, the difficulty in quantifying cooking doneness via questionnaire. As a result, dietary surveys give varying estimates of PhIP amounts that may or may not reflect actual exposures. PhIP metabolite detection in the urine of the subject who ate chicken prepared at a restaurant demonstrates that our method is sensitive enough to monitor PhIP exposure of individuals in real-life situations.

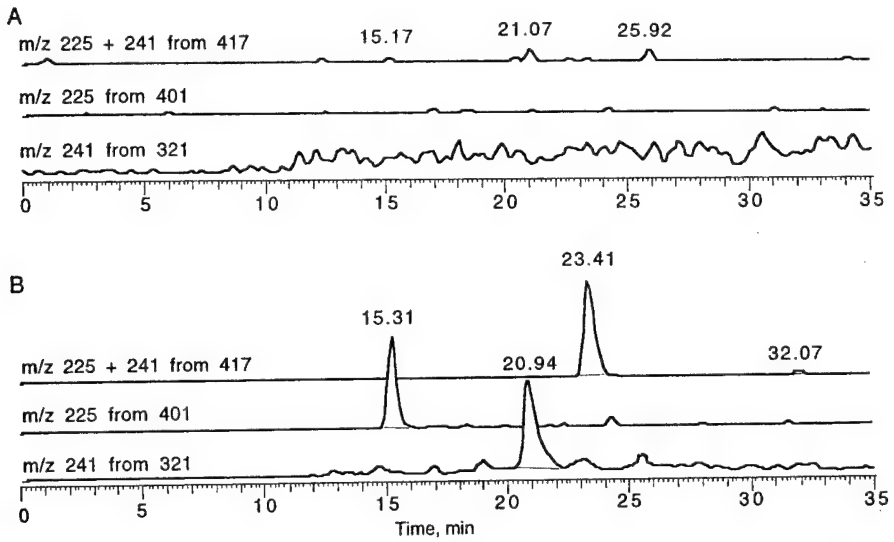

Fig. 2. LC–MS–MS chromatograms of urine from a subject abstaining from well-done meat for 24 h (A), and urine collected 0–6 h after consumption of well-done chicken (B).

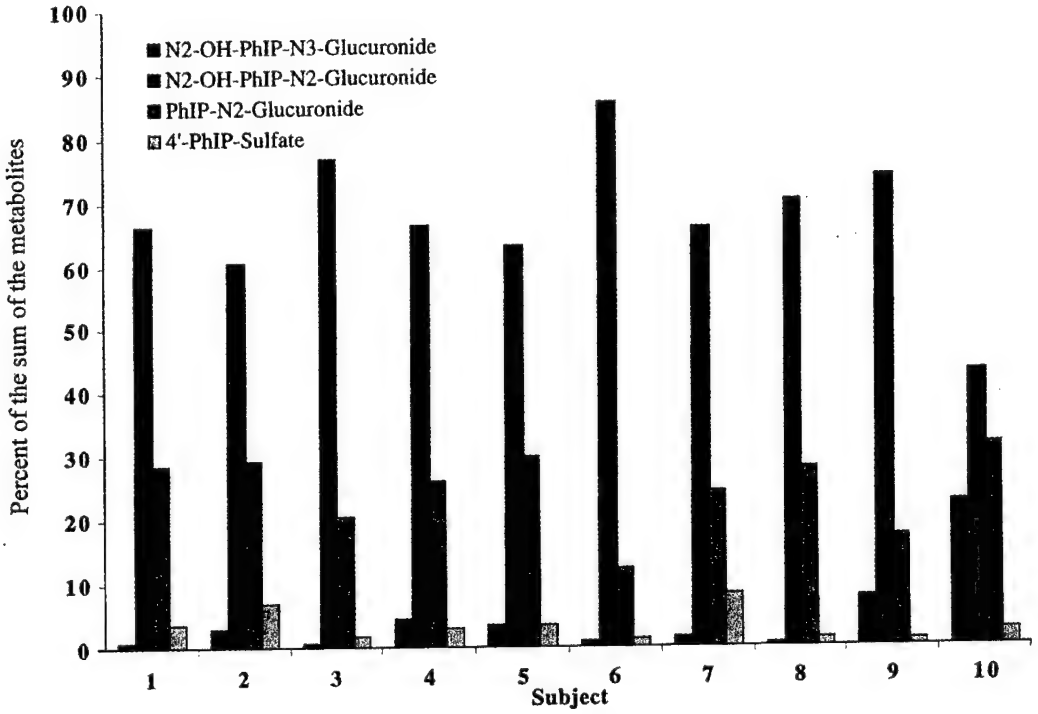

Fig. 3. Graph of individual PhIP metabolites excreted over 24 h from 10 individuals eating a single meal of well-done chicken.

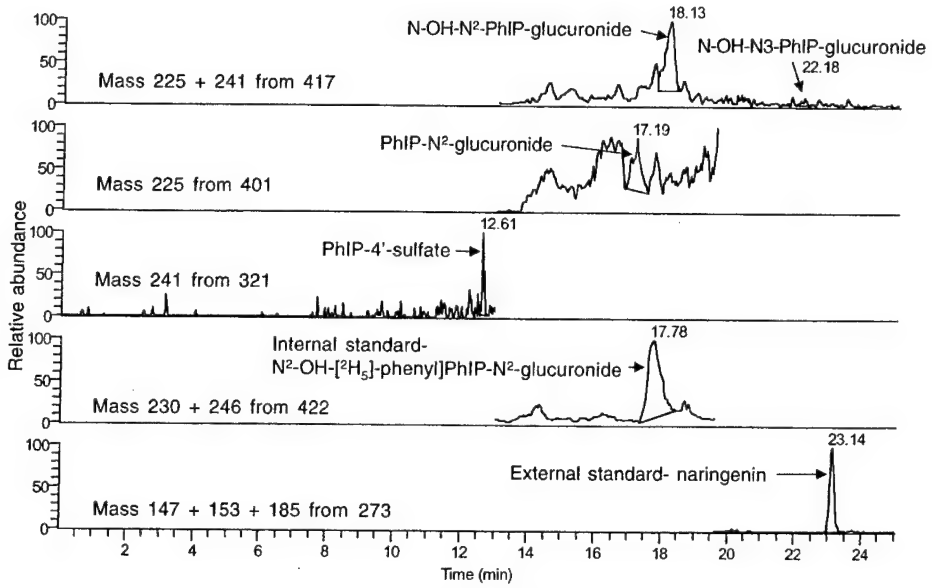

Fig. 4. LC–MS–MS mass chromatograms of urine collected after consumption of a restaurant meal of grilled chicken. Peaks identified are at the retention time of metabolites or the added internal or external standard. The equivalent of 2 ml of urine and 5 ng of internal standard were injected.

Future studies will focus on improving the method by increasing the sensitivity of metabolite detection, allowing us to lower the amount of food containing PhIP given to the volunteers. Reducing the analysis time and variation for the LC–MS–MS analysis are also needed. Repeated analysis of PhIP metabolism in the same individuals over time will help determine the consistency of PhIP metabolism, allowing us to correlate the PhIP metabolite phenotype with genotype.

Altering the metabolism of PhIP to prevent formation of biologically active species may reduce individual susceptibility and prevent the occurrence of cancers in target tissues. The method described here should make studies of individual susceptibility and dietary interventions possible in the future.

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The effect of dietary soy protein on the metabolism of PhIP in humans. M.G. Knize K.S. Kulp, and J.S. Felton, Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, Livermore CA, 94551-9900.

We devised a method to investigate the metabolism of a naturally-occurring carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), and the effect of preventive foods. Soy protein contains isoflavonoids and lignans that are implicated as protective by altering the absorption or metabolism of carcinogens. PhIP is a carcinogen formed naturally in well-cooked muscle meats. We use solid-phase extraction and LC/MS/MS to quantify PhIP metabolites in urine collected from volunteers who have eaten a meal that includes 150 g of well-done chicken cooked to contain naturally-formed PhIP. We detect four major urinary metabolites: N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, 4'-PhIP-sulfate, and N^2-OH-PhIP-N^3-glucuronide. To investigate absorptive or metabolic effects of soy protein, chicken was fed to seven male volunteers who abstained from soy protein for 4 days. Two individuals participated twice, so nine trials were recorded. Urine was collected in 6 h aliquots, and a baseline profile of PhIP metabolites before soy protein for that individual was measured. Then the subjects drank a beverage containing soy powder daily for 3 days. On the fourth day chicken was consumed again and urine collected. Preliminary results show that soy increased the relative output of the two N-hydroxylated metabolites in 7 of the 9 trials comparing pre-soy to post soy urines, suggesting the induction of P450 enzymes (P4501A1 and others) responsible for this conversion. The total output of metabolites and the rate of excretion was unchanged with soy protein intervention. These results suggest that soy protein may increase PhIP bioactivation , while decreasing detoxification metabolites PhIP-N^2-glucuronide and 4'-PhIP-sulfate metabolites. This preliminary trial did not demonstrate preventive effects for the soy protein on PhIP metabolism. (This work was performed under the auspices of the U.S. DOE by LLNL under contract no. W-7405-Eng-48 and supported by DOD Prostate Cancer Research Grand DAMD17-00-1-001grant and the NCI grant CA55861.)

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Factors affecting human heterocyclic amine intake and the metabolism of PhIP

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Abstract

We are working to understand possible human health effects from exposure to heterocyclic amines that are formed in meat during cooking. Laboratory-cooked beef, pork, and chicken are capable of producing tens of nanograms of MeIQx, IFP, and PhIP per gram of meat and smaller amounts of other heteroyclic amines. Well-done restaurant-cooked beef, pork, and chicken may contain PhIP and IFP at concentrations as high as tens of nanograms per gram and MeIQx at levels up to 3 ng/g. Although well-done chicken breast prepared in the laboratory may contain large amounts of PhIP, a survey of flame-grilled meat samples cooked in private homes showed PhIP levels in beef steak and chicken breast are not significantly different ($P = 0.36$). The extremely high PhIP levels reported in some studies of grilled chicken are not seen in home-cooked samples.

Many studies suggest individuals may have varying susceptibility to carcinogens and that diet may influence metabolism, thus affecting cancer susceptibility. To understand the human metabolism of PhIP, we examined urinary metabolites of PhIP in volunteers following a single well-done meat exposure. Using solid-phase extraction and LC/MS/MS, we quantified four major PhIP metabolites in human urine. In addition to investigating individual variation, we examined the interaction of PhIP with a potentially chemopreventive food. In a preliminary study of the effect of broccoli on PhIP metabolism, we fed chicken to six volunteers before and after eating steamed broccoli daily for 3 days. Preliminary results suggest that broccoli, which contains isothiocyanates shown to induce Phases I and II metabolism in vitro, may affect both the rate of metabolite excretion and the metabolic products of a dietary carcinogen. This newly developed methodology will allow us to assess prevention strategies that reduce the possible risks associated with PhIP exposure.

Keywords: PhIP; MeIQx; IFP; Heterocyclic amine; Food mutagen

1. Dietary intake and heterocyclic amine carcinogens

Human epidemiologic and animal studies have shown that diet has a role in the etiology of human cancer. Diet is one aspect of an individual's lifestyle that may be practically modified. Therefore, it is important to quantify dietary exposures to understand an individual's risk for cancer and to identify habits or practices that increase or decrease an individual's risk. Although complex, the interactions between the myriad different components in the whole diet may be a critical factor in determining the likelihood of cancer initiation.

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There is general consensus that potent genotoxic carcinogens are produced in meat during cooking at high temperatures. The demonstrated mutagenicity of these compounds in bacteria [1], cells in culture [2,3] and mice [4,5], support the many studies of carcinogenicity in mice and rats [1,6]. Mechanistic data show DNA adducts in rodents and humans consuming these compounds at low doses [7].

Although, the role of heterocyclic amines in cancer initiation has been well-established in animals, much less is known about the effect of heterocyclic amine exposure on tumor development in humans. The presence of heterocyclic amines in commonly consumed commercially cooked meats has been well-documented [8,9] and risk assessments made using the available data [10–12]. Depending on individual dietary and cooking preferences, human intake of heterocyclic amines may range from nanograms to micrograms per day.

2. Comparison of heterocyclic amines with other aromatic amines and the relationship to human cancer

The precedent for aromatic amines causing human cancer comes from occupational exposures in the chemical industry. In one case, all 15 workers distilling 2-naphthylamine developed bladder cancer [13]. Ward et al. showed a relative risk of 27 for bladder cancer in workers occupationally exposed to *ortho*-toluidine and aniline for greater than 10 years [14].

So, for heterocyclic amines in foods, bladder cancer might be the logical endpoint. Esophageal tumors, in addition to bladder tumors, were seen in two studies [15,16], but not in a third [17], suggesting, at least, that other tumor sites may be relevant for aromatic amine exposure.

Are the low amounts in present in some cooked meats safe because of a threshold needed to induce tumors? The doses are not known for the occupational exposures cited above, so neither the dose needed to cause the human bladder tumors, nor the difference between the occupational dose and the dietary human heterocyclic amine dose can be determined.

Gender differences are known in human bladder cancer, with males being more sensitive [18]. For well-done meat and colorectal cancer, there was a non-significant two-fold increase in males, but not in females [19]. Are mixed gender studies of aromatic amine carcinogenesis confounded? Gender differences are just beginning to be investigated in laboratory studies and need further investigation.

Recently epidemiologists have begun investigating possible links between well-done meat consumption and cancer risk. Several epidemiology studies have reported an increased risk of cancer associated with subject groups that prefer well-done meat. In 1998, Zheng et al. described a significant dose-response relationship between doneness levels of meat and breast cancer risk; women who preferred well-done hamburger, steak and bacon had a 4.6-fold greater risk of breast cancer than did women who preferred meats cooked "rare" or "medium" [20]. Other studies reported an increased risk of colorectal adenomas with increased well-done meat consumption [21,22]. Lung cancer risk has also been related to the consumption of fried, well-done meat [23]. Other studies, however, have shown either equivocal associations with well-done meat and cancers of the prostate gland [24] or negative associations with cancers of the breast [25,26], colon or rectum [11].

In all of these studies, heterocyclic amine exposure levels are based upon answers to dietary questionnaires. However, the formation of heterocyclic amines in foods depends on many cooking variables, and dietary surveys give varying estimates of heterocyclic amine dose that may or may not reflect actual exposures.

3. Prediction of heterocyclic amine intakes from dietary questionnaires

Precisely quantifying the dietary dose of heterocyclic amines in the population and individuals is essential for risk determination. The most common practice in epidemiology is to establish dietary exposure through questionnaires. These questionnaires typically use subject recall to determine the amount of meat consumed, the preparation method, and the doneness of the meat, with photographs sometimes used to estimate doneness. These parameters are all then linked to databases of heterocyclic amine content. Although these estimates of intake have been

used with the belief that they are highly precise [21], none of the current epidemiological studies are able to estimate accurate exposures, because no biomarkers of dose have been used to validate the questionnaires in any of these studies.

White meat (fish and chicken breast) has been frequently identified as a confounding factor in studies of heterocyclic amine exposure and cancer relationship. Consumption of white meats is generally associated with lower cancer rates, yet these meats have been attributed with heterocyclic amine exposures that are greater than red meats [27–29]. In an early study of laboratory-cooked chicken [30], high levels of PhIP were found in chicken samples that have not been shown to be typical in even the most well-done meat diet in our recent work. Cooking method and the interpretation of meat doneness are responsible for a great deal of variation in heterocyclic amine amounts, especially for PhIP in chicken.

For example, marinating meat is a preparation method generally not accounted for in dietary questionnaires for heterocyclic amine exposure assessment. Fig. 1 shows the formation of PhIP in chicken breast meat as a function of weight loss during cooking. Analysis was performed on meats grilled, fried, or broiled in our laboratory or on meat samples that had been sent to us previously cooked [30]. Only when chicken breast is cooked to extreme dryness (weight losses of 40% or more), do PhIP levels increase to the very high levels occasionally found. Because weight loss and the perceived dryness of the food is used as a measure of cooking doneness, it is apparent from Fig. 1 that determining when samples are "done" can have a great effect on PhIP levels. Also shown in Fig. 1 is the effect of marinating on PhIP formation. As we have described previously, marinating before grilling greatly reduces PhIP levels in chicken [31]. Notably, in samples cooked to the same degree of weight loss, PhIP levels are up to 10-fold less in the marinated samples. These results emphasize the extreme differences in PhIP levels that can occur as a result of different cooking methods.

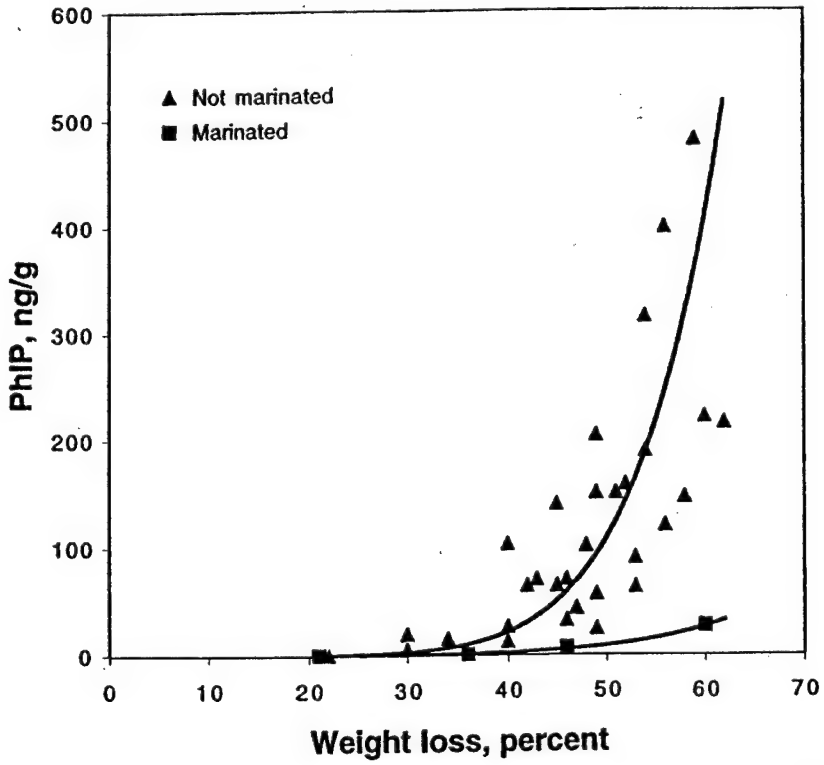

Fig. 1. The PhIP content and weight loss during grilling, frying or broiling of chicken breast. Samples marinated before cooking (squares) show low PhIP levels despite great weight loss during flame grilling.

Another uncertainty surrounds the heterocyclic amine databases used to construct exposure categories. Most epidemiologic studies of heterocyclic amines use relationships between heterocyclic amine concentrations and doneness level derived from laboratory cooking studies. However, heterocyclic amine levels in meats obtained from homes have varied considerably from the laboratory data. In a study of foods cooked under actual household conditions, grilled meat samples were obtained from households in the midwestern US. Samples were taken from volunteers responding in survey that they preferred their meat well-done or very well-done, leading us to expect high heterocyclic amine exposures in these households. Ninety-two samples of cooked meat, including 20 samples each of flame-grilled ground beef patties, pork or chicken parts and 32 samples of grilled steaks were obtained and analyzed by solid-phase extraction and photodiode-array HPLC using published methods [8].

MeIQx and PhIP values for the four different kinds of cooked meats and their averages are plotted in Fig. 2. Samples with no detectable amounts of PhIP and MeIQx were assigned a value representing half of the lowest level of detection: 0.02 ng/g for MeIQx, and 0.08 ng/g for PhIP. As expected for well-done meats, PhIP, on average, was found in greater amounts than MeIQx in each of the meat types. The biggest range of PhIP values was found in the chicken breast, undetectable levels to 48 ng/g, followed by the grilled steak and the beef patties. Pork had the smallest range of PhIP values (0–7 ng/g). The amount of MeIQx found in the samples ranged from 0 to 7 ng/g in grilled steak and chicken, 0 to 3 ng/g in grilled beef patties and 0 to 2 ng/g in pork. Surprisingly, in this collection of well-done or very well-done meats samples approximately 25% of the samples had undetectable levels of MeIQx or PhIP as shown in Table 1. Approximately 20% of the samples across all doneness categories had no detectable heterocyclic amines of any kind. Although chicken breast had some of the highest PhIP values, a comparison of PhIP levels in chicken breast and beef steak shows that the amounts of PhIP formed in the two meat types are not significantly different ($P = 0.36$) from each other.

The high variability observed in these home-cooked samples, especially for PhIP in very well-done chicken, may contribute to the contradiction of white-meat associated low cancer rates and high heterocyclic

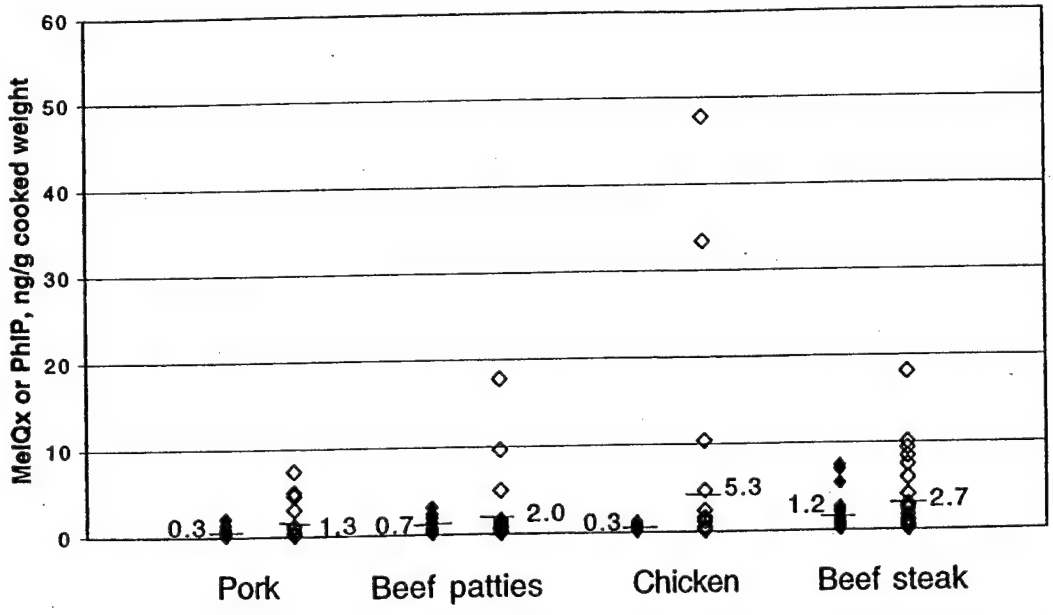

Fig. 2. Plot of MeIQx (filled symbols) and PhIP (open symbols) in grilled meat samples obtained from homes in the US whose occupants stated in a survey that they preferred their meat well-done or very well-done. Averages for each compound and meat type are shown; $n = 20$ samples for beef patties, pork, and chicken, $n = 32$ for steak.

Table 1
Percentage of samples with no detectable levels of PhIP, MeIQx or all heterocyclic amines obtained from homes specifying a preference for well-done or very well-done meats.

Meat	PhIP	MeIQx	All heterocyclic amines
Chicken	25% (5/20)	20% (4/20)	15% (3/20)
Beef steak	22% (7/32)	15% (5/32)	12.5% (4/32)
Pork	50% (10/20)	35% (7/20)	30% (6/20)
Beef patty	30% (6/20)	25% (5/20)	25% (5/20)

amine exposure. Using high heterocyclic amine values reported in an early study of laboratory-cooked chicken [30], Byron et al. concluded that chicken prepared by grilling, broiling, or pan-frying are the three foods that most reliably predict PhIP exposure [27]. However, based upon the results presented in Fig. 2, as well as analysis of meat cooked in restaurants [32], we believe that the levels of PhIP are similar in chicken and beef when the meat is cooked in typical households. In the same study by Byrne et al., broiled fish was identified as the fourth "predictor of PhIP exposure." In studies of fish cooked to the doneness usually eaten in the US or Sweden, there is little evidence in support of the conclusion that broiled fish contains more PhIP than beef steaks [33,34]. The research group that reported large amounts of PhIP in well-cooked salmon [35] found no PhIP in another grilled fish type in a follow-up study that compared laboratory grilled beef, pork (bacon), and fish [36]. Yet the latter study is not often considered when assessing dietary intake.

Based on these observations it is apparent that quantifying human heterocyclic amine exposure is not a simple task. Formation of heterocyclic amines in meat during cooking is highly dependent upon cooking method and doneness levels. Individual exposure depends upon meat consumption patterns. The compelling conclusion from these meat and cancer studies is that humans may be exposed to genotoxic carcinogens over a lifetime. Intake levels are low; still, one microgram of MeIQx (a 200 g steak with 5 ng/g) has 2.8×10^{15} molecules that can be absorbed, and then activated or detoxified through metabolism. Clearly, focusing on just doneness level simplifies efforts to estimate heterocyclic amine exposure but will overlook important variables such as cooking practices and meat type.

4. Human PhIP urinary metabolites as a measure of metabolism phenotype

The enzymes known to be involved in the metabolism of heterocyclic amines are found at a variety of levels and activities within the human population [37]. Variation in the expression of these enzymes suggests variation in the amounts of the activation compared to the detoxification intermediates produced. Changes in the activity of these enzymes can occur due to changes in lifestyle habits and diet. Altering the metabolism of heterocyclic amines by altering the activity of metabolizing enzymes may prevent formation of biologically active species and thus may prevent the occurrence of cancer.

One way of monitoring human metabolic activation/detoxification patterns and possibly identifying individuals that may be more or less at risk for cancer initiation is through measuring the excretion of heterocyclic amine metabolites in the urine. Identifying and quantifying metabolites produces a "snapshot" of recent exposure as well as a way to monitor changes in metabolic enzyme activity. We developed a method for quantifying PhIP metabolites in human urine following a single meal of well-done meat.

Pioneering work in in vivo human metabolism examined the relationship between urinary excretion of the unmetabolized parent compound and the dose received in well-done hamburgers [38,39]. Other studies demonstrated the presence of PhIP and PhIP conjugates in human urine, but in these studies the urine was first treated with acid to hydrolyze the Phase II metabolic conjugates to the parent amine. These investigations showed that PhIP is bioavailable in humans, but did not give information about specific metabolic pathways [40–42].

Most recently, specific results about the identity of human PhIP metabolites were obtained in studies that investigated human PhIP metabolism following administration of [^{14}C]-labeled PhIP to patients undergoing cancer surgery [43–45]. Surprisingly, the relative amounts of human urinary metabolites were unlike those of rodents and more like those of dogs [44]. These studies identified four major human PhIP metabolites: N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, PhIP-4′-sulfate, and N^2-OH-PhIP-N^3-glucuronide. Based on the metabolite identification, we developed a solid-phase extraction, LC/MS/MS

method that quantifies the four known PhIP metabolites in human urine, following a single meal of well-cooked chicken [46]. Chicken is used in this assay because we can produce PhIP in overcooked chicken without a concomitant amount of other known heterocyclic amines. Because the PhIP is formed naturally in the chicken at levels that represent possible dietary exposures, we can apply this method to characterize PhIP metabolism in normal, healthy volunteers.

To determine the feasibility of affecting PhIP metabolism by dietary supplementation with a putative chemopreventative food in humans, we investigated the effect of broccoli on PhIP metabolism by quantifying changes in PhIP urinary metabolites. The study protocol was reviewed and approved by the Institutional Review Board for Human Research at Lawrence Livermore National Laboratory, and informed consent was obtained from each subject prior to beginning the study. The individuals participating were recruited from the local workforce, were in good health, non-smokers and of normal weight. The meat was prepared by cutting boneless, skinless chicken breasts into approximately 2.5 cm pieces and pan-frying for 25–35 min. The chicken pieces generally lost 40% of their weight during cooking, and at the end of the cooking time the chicken appeared white with some browning. A representative sample was removed and analyzed for PhIP content using published methods [8]. Volunteers were provided with 150 g of chicken containing from 60–90 ppb PhIP for a total dose of 9–13 μg.

In this preliminary study, we fed six human male volunteers well-cooked chicken, collected urine, and measured a baseline urinary PhIP metabolite profile. We then gave the subjects one cup of steamed broccoli daily for 3 days. On day 4 we fed them chicken again and collected urine. Three of the individuals have repeated the procedure at 3-month intervals. Volunteers were asked to not eat grilled meat for 24 h prior to eating the chicken meal and to abstain from broccoli and related cruciferous vegetables for 3 days before the intervention. No further dietary restrictions were imposed. All subjects were provided with other non-meat foods and beverages with the cooked chicken. Control urine was collected before eating the chicken and samples were collected for 24 h after in increments of 6 h.

Urine samples were prepared according to Kulp et al. [46]. Briefly, an internal standard of deuterium labeled *N*-OH-PhIP-N^2-glucuronide was added to 5 ml samples of urine. The urine was then applied to a pre-conditioned macroporous polymeric column. Metabolites were eluted with methanol and the methanol fraction evaporated to dryness under nitrogen. The metabolites were re-dissolved in 0.01 M HCl and high molecular weight contaminants were removed by filtering the solution through a centrifugal filter at 3000 × *g* overnight. The filtrate was applied to a pre-conditioned benzenesulfonic acid column and the column washed with a mixture of methanol and 0.01 M HCl. The metabolites were eluted onto a coupled C18 column with 0.05 M ammonium acetate, pH 8. The C18 column was washed with 5% (v/v) methanol/H_2O and eluted from the C18 column with 50% (v/v) methanol/H_2O. The metabolites were dried under nitrogen and 1 ml urine equivalents were injected into the LC/MS/MS in a volume of 20 μl.

Metabolites were detected with an ion trap MS (model LCQ, Finnigan, San Jose, CA) in the MS/MS positive ion mode using an electrospray interface as published [47]. Alternating scans were used to isolate $[M + H]^+$ ions at mass 417, 401, and 321 for natural PhIP metabolites, and 422, for the pentadeutero-labeled internal standard metabolite. Collision energy was 25%. Daughter ions were detected at appropriate masses: 241 (M + H-glucuronic acid) and 225 (M + H-glucuronic acid-OH) from 417 for the *N*-OH-N^2 and N^3-glucuronide, respectively, 225 (M + H-glucuronic acid) from 401 for the PhIP-N^2-glucuronide, 241 (M + H-SO_3) from 321 for PhIP-4′-sulfate, and 246 (M + H-glucuronic acid) and 230 (M + H-glucuronic acid-OH) from 422 for the internal standard, *N*-OH-(D_5-phenyl)PhIP-N^2-glucuronide.

In Fig. 3, the rate of the excretion of PhIP urinary metabolites is shown. These data illustrate that with the exception of volunteer 3, the volunteers excreted more metabolites during the first 6 h after the broccoli intervention compared to the baseline level determined 1 week prior. Individual differences seen at different times may be due to differences in diet, which was uncontrolled except for the cooked meat and broccoli ingestion. Broccoli contains isothiocyanates, which have been shown to induce both cytochrome P450 enzymes

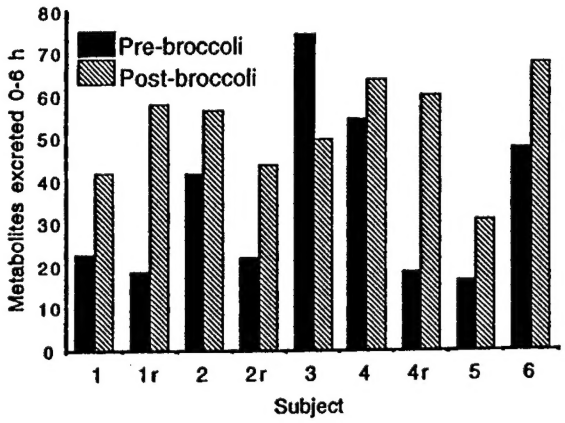

Fig. 3. Percent metabolites excreted in the first 6 h after eating chicken in six human volunteers either after abstaining from broccoli for 3 days (pre-broccoli) or after eating at least one cup of cooked broccoli for 3 days (post-broccoli). Three individuals were assayed twice; "*r*" signifies repeat. Five of six individuals excreted more of the metabolites after broccoli consumption, implying induction of metabolizing enzymes.

and glucuronyl transferases [48]. Our data suggest that broccoli may be affecting the rate of PhIP metabolism, because of the increase in the fraction of metabolites excreted in the 0–6 h time period.

There are many foods implicated in inducing or inhibiting carcinogen metabolizing enzymes. The PhIP urinary metabolite assay described above is designed to determine the influence of such foods on a dietary carcinogen at dietary doses in people. Because the metabolites are present in the urine at nanograms per milliters levels the assay poses several analytical difficulties. Extensive sample clean-up must be done to identify and quantify the metabolites above the background inherent in the urine sample and to prevent HPLC column degradation. The assay can still be improved in several ways. Heavy-isotope labeled metabolites are necessary for recovery determination of the *N*-OH-N^3-PhIP-glucuronide, PhIP-N^2-glucuronide, and PhIP-4′-sulfate. Additional PhIP metabolites are known to be present in human urine but have not yet been fully characterized. Although the unknown metabolites occur in smaller amounts than the four detected, quantifying these metabolites would provide a more complete picture of biological fate of the PhIP ingested in the chicken meal. Recently available mass spectrometers have about 10-fold more sensitivity than the current model, which might lead to improved peak signal, thereby reducing injection-to-injection variability.

5. A biomarker of heterocyclic amine exposure is still needed

To understand the effect of heterocyclic amine exposure on human health, we need to be able to assess actual exposures from meat prepared as it is commonly eaten in homes. Although measuring urine metabolites is one way of characterizing metabolism patterns, the metabolites excreted in the urine only represent exposures that may have occurred in the previous 24 h. The optimal biomarker of exposure would integrate heterocyclic amine exposures over time. Hair has been investigated as a marker of PhIP exposure over the previous 6 months [49].

Aflatoxin exposure assessment presents complexities similar to the heterocyclic amine exposure assessment. It sometimes occurs in only some foods, so the food contamination and amount eaten are both important for dose determination. A biomarker of exposure is available for aflatoxin, but a dietary questionnaire showed no positive correlation with measurement of blood serum levels of the AFB1-albumin adduct [50]. A biomarker would help judge if dietary questionnaires are useful for determining heterocylic amine intake. But a questionnaire and biomarker measurement are contemporary. Perhaps what is really needed is data regarding intake 20 years ago for individuals, or perhaps the heterocyclic amine intake during more sensitive adolescent years.

6. Conclusions

Intake variation of heterocyclic amines is suggested to be three orders of magnitude above the limit of detection from restaurant data for steaks [32] in the US. This seems to be a useful range in which to group human exposures and their cancer incidences.

The idea that the "the dose makes the poison" is important and may be relevant for dietary exposures to carcinogenic heterocyclic amines. It is possible that all heterocyclic amine doses are below the dose needed to show an effect. However, no evidence for threshold effects or non-linearity of DNA adducts exists for MeIQx in either rodents or humans [51].

The goal of understanding and reducing cancer is worthwhile. It requires understanding the tumor initiating mechanisms and controlling the relevant influences in epidemiology investigations. The heterocyclic amines are the perfect model compounds for both the basic and applied research, and results can be directly transferred to humans. The data are not currently available to fully characterize the relationship between heterocyclic amines and human cancer.

Acknowledgements

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